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An Examination of Prostaglandins as Causative Agents in Pine Needle (Pinus ponderosa) Abortion

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AN EXAMINATION OF PROSTAGLANDINS AS CAUSATIVE AGENTS IN PINE NEEDLE (PINUS PONDEROSA) ABORTION

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IN PINE NEEDLE (PINUS PONDEROSA) ABORTION

This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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INTRODUCTION

Cow and calf losses due to pine needle abortion in cattle grazing foothill ranges of ponderosa pine (Pinus ponderosa) continue to cause managerial and economic problems for ranchers in South Dakota, Colorado, Idaho, California, Montana and many areas throughout Canada. Because of its overall economic significance, investigators have been attempting to isolate and characterize causative agents of pine needle abortion. To date, a large number of pine needle constituents have been shown to possess abortifacient capabilities. In addition to actual pine needles, it appears that several other undefined factors may be associated with, and(or) predispose animals to, pine needle abortion. These factors are (1) stage of gestation when pine needles are consumed, (2) environmental stresses, (3) animal condition and (4) general physiology of the animal.

These undefined potentiating factors represent a major stumbling block in pine needle abortion research, because it has thus far been impossible to consistently induce abortions in cattle under experimental conditions. Without this documentation, it has been difficult to clearly delineate pine needle abortion from other abortive disorders. In order to accurately define pine needle abortion, it will be necessary to isolate, identify and test all possible pine needle constituents having potential abortifacient capabilities. Furthermore, the mode of action of these causative agents in mammals must be determined.

The recent discovery that prostaglandins or prostaglandin-like compounds are contained in ponderosa pine needles raises the possibility

of their involvement in pine needle abortion. The luteolytic and abortifacient capabilities of some prostaglandins have received considerable attention due to their use in estrus synchronization and the termination of unwanted pregnancies.

The purpose of this study was to investigate the possibility of prostaglandins as causative agents in pine needle abortion. The objectives were (1) to investigate the effects of known prostaglandins and a diterpene resin acid on pregnant mice and to establish dose-response relationships, (2) to use established baseline dose-response data for comparison in testing the biological activity of three separate pine needle fractions for prostaglandin activity in causing abortions in pregnant mice during late gestation, (3) to produce experimental abortions in cattle during the third trimester of pregnancy by feeding ponderosa pine needle diets, (4) to document gross pathological changes in maternal and fetal systems following abortions and (5) to monitor blood serum progesterone levels and leukocyte numbers throughout the cattle experiment in an attempt to differentiate between a hormonal imbalance, infectious processes and(or) toxic reactions as the principal mode of action for causative agents in pine needle abortion.

REVIEW OF LITERATURE

Potential Causative Agents in Pine Needle Abortion

Early investigations of pine needle abortion centered on the possibility of an anti-estrogenic factor as a causative agent. This research employed a bioassay technique using immature, female mice and rats. Extracts of ponderosa pine needles were fed or injected into experimental animals. The activity of the pine needle extract was then assessed by the decrease of gross uterine weight and expressed as a percentage of body weight (Cook, 1960; Allen and Kitts, 1961; Allison and Kitts, 1964; Cook and Kitts, 1964; Chow et al., 1972).

Dorfman (1962) defined anti-estrogenic substances as compounds which interfere with any action of an estrogen following its secretion into the general circulatory system. He further stated that inhibition of estrogen action has been shown for androgenic substances, progestin substances, estrogens and corticoids. Although Dorfman did not discuss substances which interfere with normal gonadotropin levels, such substances are able to interfere with estrogen secretion so that the end result is of a similar nature, i.e., anti-estrogenic (Cook, 1960).

In experiments conducted by Cook (1960), ponderosa pine needle extracts were found to contain one or more factors which had anti-estrogenic effects on the reproductive system of mice and rats. Oral administration of a water-soluble fraction of a crude acetone extract decreased the uterine weight of immature mice and disrupted the estrous cycles of adult female rats. When injected sc into female rats, this

extract reduced the uterine response to an estrogen injection, with the reduction of uterine weight occurring in less than 6 h.

Allen and Kitts (1961) fed an acetone and ether-soluble pine needle extract to immature female mice and 4-d pregnant mice. These investigators found that the acetone fraction of yellow pine needles (Pinus ponderosa) contained factor(s) that depressed the uterine weight of immature mice, while the ether fraction contained compounds that were toxic to mice. Both acetone and ether extracts caused a depression of the metabolism of weanling mice, contributed to embryonic mortality and reduced fetal weight.

Further investigations on the anti-estrogenic effect of ponderosa pine needles (Cook and Kitts, 1964) showed an active factor to be soluble in acetone, ethyl acetate and ethanol as judged by reduced uterine weight upon oral and parenteral administration to immature rats and mice. The solubility of the anti-estrogenic factor in ethanol was verified by Allison and Kitts (1964). In this experiment, sc injections of an ethanol extract of pine needles decreased uterine weights in rats during a 6-h bioassay. In addition, a chloroform extract of the parent ethanol extract also decreased uterine weights in immature mice which had received priming doses of estrone and estradiol 17β.

Chow et al. (1972) tested abortifacient activity of three separate pine needle extracts corresponding to water-soluble, acetone-soluble and ether-soluble (volatile) fractions. The water-soluble fraction when fed to immature mice showed the most pronounced depression of uterine growth and also had the most detrimental effect on reproduction.

Conclusions from this study indicated the active agent in ponderosa pine needle extracts to be water-soluble, to accumulate with duration of feeding and to have no adverse effects on fertilization and implantation. According to results of Cogswell (1974), pine needle components detrimental to normal embryo development of mice and rats were present in both water-soluble and acetone-soluble fractions.

During the following 2 yr, aqueous fractions of pine needles prepared by Chow et al. (1972) caused little reproductive failure in mice. This inconsistency in activity raised doubts concerning the toxicity of the pine needles and prompted Chow et al. (1974) to conduct a preliminary study to determine whether metabolites produced by fungi of pine needles could cause reproductive failures. Fungi incubated in an aqueous pine needle fraction for 2 d at room temperature showed a significantly greater disruptive effect on reproduction when fed free choice to pregnant mice on d 1 to 4 of pregnancy than did the pine needle aqueous fraction without fungi. In order to substantiate the hypothesis that mycotoxins instead of pine needles were the cause of reproductive failure, another experiment was undertaken. In this experiment, the aqueous fraction was autoclaved at 110 C for 15 min to inactivate the toxic agents (Chow et al., 1972). This inactivated aqueous fraction was then used as the medium for the growth of fungi isolated from pine needles. Pregnant mice fed this reactivated aqueous-fungi preparation on d 1 to 7 of gestation showed fetal resorptions in four of eight mice tested.

Anderson and Lozano (1977) reported that feeding autoclaved ground pine needle fiber remaining after water extract to 5-d pregnant mice resulted in fetal resorptions. These results suggested that abortions could be produced by a heat stable toxin insoluble in water. Of the mice fed water-soluble extracts, the lowest pregnancy rates were observed in those groups in which water extracts were incubated for 2 wk, suggesting the presence of mycotoxins. Although no adult mortality occurred with water extract preparations, there were weight losses in each group. The physical condition of treated mice when compared to control animals was considered good. Total food consumption between extract-treated mice and control mice was constant. In contrast, treatment groups fed pine needle fiber had a high adult mortality, with those surviving having diarrhea and poor physical conditioning. Mice receiving pine needle fiber treatments incubated for 2 wk had a lower feed intake compared with other treatment groups, and histopathological examinations revealed severe depletion of fat storage depots indicative of starvation.

In a continuation of their investigations, Anderson and Lozano (1979) determined that the heat stable toxin observed in the previous experiment (Anderson and Lozano, 1977) was soluble in several organic solvents. On d 4 to 10 of gestation, pregnant mice were fed diets containing pine needle fiber extracts prepared from methanol, chloroform, hexane or 1-butanol. Separate diets containing the corresponding autoclaved solvent extracted fiber were also fed. By monitoring the number of embryo resorptions following consumption of solvent-extracted fiber, it was concluded that the heat stable toxin was most soluble in ethanol

and least soluble in 1-butanol. Although feed wastage was not measured in this study, a maximum embryo resorptive dose (ERD_{50}) for fresh green pine needles was determined to be 1.49 $g \cdot mouse^{-1} \cdot d^{-1}$ and 1.08 $g \cdot mouse^{-1} \cdot d^{-1}$ for autoclaved green pine needles. Feeding a half ration (filter paper/ purina mouse chow) to pregnant mice did not lead to reproductive failure. Upon histological examination, there was no evidence of depleted fat storage depots. Twenty unbred females were used in a weight loss study during the second part of this experiment. Mice fed an ethanol extract lost 29% of their initial body weight over the 6-d feeding period, whereas pregnant mice in the first part of the experiment also resorbed their embryos. By contrast, pregnant mice fed the ethanol-extracted fiber did not resorb their embryos, yet unbred mice fed the same diet still underwent a 12% loss in body weight. Based on these weight loss data (total feed consumption was not measured), these authors concluded that the weight loss observed in mice fed pine needle fiber extracts was not due to lack of nutrients but rather to some toxic factor present in ponderosa pine needle fiber.

In an attempt to determine the nature of the toxic heat-stable component of ponderosa pine needle fiber (Anderson and Lozano, 1977, 1979), Kubik and Jackson (1981) conducted fractionation studies of a hexane extract of ponderosa pine needles using silicic acid column chromatography. The embryotoxic effect of individual fractions was tested by administering fractions via stomach tube to pregnant mice on d 1 to 5 of gestation. The column fraction accounting for the greatest percentage of fetal resorptions (75%) was determined through gas-liquid

chromatography to contain seven individual diterpene resin acids. The ERD_{50} determined following administration of the total resin acid mixture was calculated to be 22.4 mg of resin acid·mouse^{-1}·d^{-1} or approximately 2 g of needles·mouse^{-1}·d^{-1}. These results differed somewhat from the ERD_{50} of 1.49 g of fresh green needles·mouse^{-1}·d^{-1} and 1.08 g of autoclaved green needles·mouse^{-1}·d^{-1} during d 5 to 10 of gestation as reported by Anderson and Lozano (1979). It was not concluded from this experiment if the isolated diterpene resin acids acted synergistically, if the embryotoxic activity was dependent on the concentrations of particular acids or if activity was due to specific compositions of resin acids present. A general toxicity to resin acids as a mode of action for embryo resorption was dismissed by these authors, since pine needles of other conifer species do not have this effect on reproduction. The possibility of diterpene resin acids being the only heat-stable embryotoxin is doubtful since Anderson and Lozano (1979) indicated methanol- and hexane-extracted fibers to be slightly more toxic to pregnant mice than the ethanol-extracted fibers. Also, the embryo toxicity of the hexane-extracted ground needle fiber/lab blox ration when fed ad libitum on d 1 to 7 of gestation resulted in 18% embryo resorptions which was slightly above the 13% embryo resorptions of the control mice receiving only the lab blox ration.

The concept of microorganisms first suggested by Chow et al. (1974) and later by Anderson and Lozano (1977) as possible causative agents in pine needle induced fetal resorptions in mice was further investigated by Adams et al. (1979). This study was conducted to

determine if (1) an infectious microorganism could be isolated from the bloodstream of mice in the pre- and post-aborting time period when the animals were fed a diet of ponderosa pine needles and (2), if isolated, could this microorganism cause the same pathological abnormalities as a diet of ponderosa pine needles. An infectious microorganism identified as Listeria monocytogenes was isolated from the bloodstream of 4-d and 9-d pregnant mice. Following injection of the isolate, fetal resorptions occurred. Upon histological examination following abortion, speckled livers, spleen atrophy and hemorrhagic intestines were observed.

Factors that cause and perpetuate listeriosis have not been fully delineated. Furthermore, it is not known why Listeria monocytogenes can cause such a variety of disease forms including encephalitis, meningitis, septicemia, abortions, liver diseases and papular skin lesions (Murray et al., 1926). Listeria-induced abortion in cows is a very serious and potentially fatal malady with most abortions occurring during the last trimester of pregnancy. Some calves are born alive but weak, and cows having late abortions frequently have pyrexia, depression, retained placentas and purulent genital exudates. In pregnant animals, Listeria monocytogenes appears to have an affinity for fetoplacental tissues, especially during the last trimester of pregnancy. Infected fetuses may die and be retained in utero for 24 to 72 h before being expelled or, if infected near term, may be born alive but weak (Weis and Seeliger, 1975).

These pathological changes in Listeria-induced abortion were similar to those observed by Stevenson et al. (1972) in pine needle-induced abortions. Pine needle abortion was characterized by excessive uterine

hemorrhaging, a characteristic nauseating odor, septic metritis and peritonitis as consistent entities. Furthermore, pine needle-induced abortions in cows are likely to occur within 48 h to 2 wk after consumption of pine needles and are associated with weak contractions and incomplete dilation of the cervix. If the cow is approaching term at the time of pine needle consumption, the calf, if born alive, is usually very weak at birth. A persistent retained placenta, atonic uterus filled with uterine fluid and placental debris and blood were also constant findings. In a separate study, James et al. (1977) reported that necropsy of fetal tissue expelled after the consumption of pine needles revealed pronounced necrosis of proximal convoluted tubules of the kidney, pulmonary congestion and excessive hemoglobin breakdown.

Neff et al. (1982) further documented the pathological effects of pine needles in pregnant mice and determined the time sequence of reproductive dysfunction during pine needle ingestion throughout gestation. Air-dried ground pine needles were fed to pregnant mice during early and late gestation with representative mice killed from each group daily to determine stages of embryonic loss. Food consumption was monitored daily with control animals receiving the same quantity of diet as experimentals. Results showed the expression of the active pine needle factors to be dependent upon the precise period of gestation when animals ingested pine needles. As observed in an earlier study (Anderson and Lozano, 1979), all groups of mice experienced a weight loss that could not be attributed to a reduced caloric intake. A

reduction in viable embryos was most evident on d 8 of pine needle consumption for those mice fed on d 1 to 10 of gestation, although fetal resorptions were also noted on d 6, 7, 9 and 10. Positive pontamine blue assays substantiated that the initial stages of implantation were complete prior to fetal resorptions, confirming earlier observations by Chow et al. (1972). Toxic reactions to the pine needle diet early in gestation were evident only during the first 2 d of feeding. Mice exhibited some loss of coordination and were lethargic. These symptoms subsided by the time of examination on d 10 of gestation and no gross pathological changes were observed. In contrast, mice receiving the pine needle diet from d 10 to 20 of gestation showed substantial pathological changes. Maternal and fetal mortality were observed with high incidence manifesting itself primarily after the 15th day of gestation. Vaginal bleeding, an oily coat condition, general lethargy and loss of coordination were apparent throughout this ingestion period. Upon dissection of the viscera, a characteristic pungent odor was detectable. The animals possessed blood-filled intestines and mottled livers and kidneys. The most dramatic changes involved a decrease in thymus and spleen weights following 2 d of diet consumption at d 12 of gestation and an increase in adrenal weight observed 4 d later. The increase in adrenal weight corresponded to the period of highest incidence of fetal and maternal mortality. During this period, adrenal glands became red and swollen, with the increase in weight due to an increase in the medullary component of the gland. Non-pregnant females fed the same pine needle diet did not exhibit these

morphological changes following 10 to 21 d on the pine needle diet, although initial signs of lethargy and loss of coordination were observed. Results from this study not only serve to document gross pathological changes that occur in the mouse maternal system upon ingestion of ground pine needles during early and late stages of pregnancy but also, upon comparison with the effect of pine needles on the bovine system (Stevenson et al., 1972), give credence for the use of the mouse model in the study of pine needle abortion.

Recently, Manners et al. (1982) examined a water-soluble fraction of an acetone extract of ponderosa pine needles and isolated via chromatographic methods seven lignol compounds not previously reported. Two of the isolated compounds were structurally similar to a water-soluble 2,3-dihydrobenzofuran derivative, lithospermic acid, isolated from Lithospermum ruderale roots (Kelly et al., 1975). Cranston and Robinson (1949) reported that a 50% ethanol extract of Lithospermum ruderale decreased frequency of littering in mice, caused prolonged diestrus in mice with previously regular estrous cycles and caused decreased weights of sex organs, thymus glands and pituitary glands. In separate experiments by Zahl (1948), estrous cycles in mice returned to normal when a lithosperm experimental diet was replaced by a control diet. Injection of lithosperm plant extracts into rats did not inhibit the action of administered estradiol 17β, ovaries and uteri failed to mature and luteal development was absent (Noble and Plunkett, 1950; Plunkett et al., 1950).

The biological activity of natural dilignols in large animals has yet to be verified. However, structurally related dibenzylbutrolactone lignans, recently isolated from human (Setchell et al., 1980b) and veret monkey urine (Setchell et al., 1980a), appear to be associated with luteolytic activity and the regulation of the length of the luteal phase of the menstrual cycle. The verification of these compounds is limited by their natural availability, and initial bioassay experiments may be restricted to the examination of more readily available compounds with closely related structures such as dihydrobenzofuran derivatives like lithospermic acid.

In review, five possible categories in which potential causative agents in ponderosa pine needles could be classified include (1) water-soluble and heat labile (Chow et al., 1972; Cogswell, 1974); (2) water-insoluble and heat stable (Anderson and Lozano, 1977, 1979; Kubik and Jackson, 1981); (3) fungal mycotoxins (Chow et al., 1974; Anderson and Lozano, 1977); (4) infectious microorganisms such as Listeria monocytogenes (Adams et al., 1979) or (5) water-soluble and heat stable, such as lithospermic acid (Manners et al., 1982). Upon closer evaluation of the pine needle extraction procedures and extraction solvents used by these investigators, this distinct categorization between possible causative agents becomes less evident. Due to the polar to nonpolar extraction capabilities of solvents such as acetone, ether and ethyl acetate used in the isolation of water-soluble fractions of acetone extracts of ponderosa pine needles (Allen and Kitts, 1961; Cook and Kitts, 1964; Chow et al., 1972; Manners et al., 1982), a potential overlap

between these extracted pine needle constituents and water-insoluble fractions obtained by Anderson and Lozano (1979) and Kubik and Jackson (1981) should be considered.

It seems logical that consumption of toxic agents such as diterpene resin acids (Kubik and Jackson, 1981) in large enough quantities would have an adverse effect on an animal's physiological well-being. However, it is still unknown whether this toxic action is the actual cause of observed reproductive failure following ingestion of ponderosa pine needles or simply a predisposing factor to yet other unknown pine needle constituents.

The possibility of ponderosa pine needles containing specific luteolytic compounds was first suggested by Cook (1960) and more recently by Manners et al. (1982). In the following section, the potential luteolytic nature of another pine needle constituent, prostaglandins, will be reviewed as known abortifacients and as possible causative agents in pine needle abortion.

The Association of Prostaglandins With Pine Needle Abortion

In 1979, Attrep et al. isolated and identified prostaglandin A_1 from onions. This was a significant discovery since up to this time prostaglandins had been isolated only in animal species. In radioimmunoassay studies by J. B. Lee (personal communication), prostaglandin E_2 (PGE_2) was identified in ponderosa pine needle extracts. Presently, M. Attrep (personal communication) is screening pine needle fractions for the presence of other prostaglandins and has also purified fractions corresponding to prostaglandin E, F and perhaps prostaglandin A.

The luteolytic and ultimate abortifacient capabilities of certain prostaglandins, particularly prostaglandin $F_2\alpha$ ($PGF_2\alpha$), has been confirmed in experiments utilizing laboratory animals (Bartke et al., 1972; Labhsetwar, 1972; Marley, 1972; Persuad, 1974). In experiments by Marley (1972), sc injections of $PGF_2\alpha$ and PGE_2 terminated pregnancy in 5-d pregnant mice, while similar injections of PGE_1 had no effect. In the same experiment, $PGF_2\alpha$ caused fetal resorptions in ovariectomized 5-d pregnant mice receiving pituitary homogenate to maintain pregnancy. It was concluded from these experiments that $PGF_2\alpha$ exerted its anti-fertility effects by antagonizing the action of gonadotrophic hormones in the ovary. Labhsetwar (1972) found 100 µg $PGF_2\alpha$ to be effective in terminating pregnancy in mice when injected sc once daily from d 4 to 6 of gestation. In another study, a single sc injection of 300 µg $PGF_2\alpha$ on d 4 of gestation interrupted pregnancy in seven of 10 female mice, with mice returning to estrus 6 to 8 d after the first mating (Bartke et al., 1972). Goyings (1979) reported a decrease in pregnancy rate and mean number of fetuses per litter at oral administration of $PGF_2\alpha$ at 20 mg/kg or 600 µg/30-g rat. $PGF_2\alpha$ when administered after midgestation in mice (d 10) was also found to cause fetal resorptions with the frequency of intrauterine death dependent on the route of administration, the day of treatment during gestation and the dose of prostaglandin administered (Persuad, 1974). Neither ovaries nor placentas of treated animals showed any morphological changes indicative of damage. Those fetuses surviving to term following maternal treatment with $PGF_2\alpha$ were normal and showed no developmental defects.

The use of $PGF_2\alpha$ as a luteolytic agent in cattle has recently received considerable attention due to its application in the control and synchronization of estrus. In many domestic species, estrus and ovulation are suppressed during the greater part of the cycle (the luteal phase) by progesterone secreted by the corpus luteum (Cooper and Walpole, 1975). Only after the corpus luteum has reached the end of its functional life span will the animal again come into fertile heat. The induction of luteal regression before the time when it would normally occur is followed by the appearance of estrus and ovulation. By treating animals during the luteal phase of the estrous cycle with a luteolytic agent such as $PGF_2\alpha$, it is possible to bring a large percentage of animals into estrus at will. Estrus synchronization has many advantages to the livestock industry. One of the major advantages is that of facilitating the use of artificial insemination. In turn, artificial insemination is of value in the genetic improvement of livestock, in the control of disease and potentially in terms of convenience and cost.

After administering an effective dose of $PGF_2\alpha$ to cows in the luteal phase of the estrous cycle, the corpus luteum is reduced in size within 24 h and by 72 h is impalpable (Louis et al., 1972). Blood progesterone falls by about half within 4 to 6 h and within 48 h may be below the limit of detection (Liehr et al., 1972; Inskeep, 1973; Chenault et al., 1976), where it remains until after ovulation.

It is well known that cattle require an ovarian source of progesterone throughout the greater part of pregnancy to maintain the gravid uterus. Enucleation of the corpus luteum (McDonald, 1953) or

ovariectomy (Erb et al., 1967) before about the 200th d of gestation leads to abortion. Prostaglandin-induced luteolysis during pregnancy produces the same result as enucleation or ovariectomy and has been shown to induce abortion in cattle up to 150 d of gestation (Lauderdale, 1972; Lamond et al., 1973; Millar, 1974; Refsal et al., 1976). Lamond et al. (1973) produced abortion in two cows by infusing 28 mg of $PGF_2\alpha$ directly into the uterine artery, while Millar (1974) reported that 15 heifers 29 to 52 d pregnant aborted following sc administration of 30 mg $PGF_2\alpha$. Brand et al. (1975) aborted 10 of 10 heifers pregnant 2 to 5 mo with two daily im injections of 25 mg $PGF_2\alpha$. Refsal et al. (1976) produced abortion in 20 heifers during the first trimester of pregnancy with a single im injection of 40 mg $PGF_2\alpha$, while a single im injection of cloprostenol (a synthetic analogue of $PGF_2\alpha$) was found to be equally effective in causing abortion in feedlot heifers up to 150 d of gestation using dosages of 62.5, 125, 250, 375 and 500 μg of cloprostenol (Copeland et al., 1978).

Retained placentas are a frequent consequence of induced parturition in cows treated with exogenous $PGF_2\alpha$ (Zerobin et al., 1973; Henricks et al., 1977; Plenderleith, 1978). These studies emphasize the fact that, under normal physiological conditions, delivery of fetus and placenta are well synchronized and that desynchronization by prostaglandins delivers the fetus but not the placenta (Zerobin et al., 1973).

The potential for prostaglandins other than $PGF_2\alpha$ to have luteolytic and abortifacient capabilities has so far been reported only in the prostaglandin E series. PGE_2 has been widely used for termination

of pregnancies in women during the first and second trimester of pregnancy (Karim, 1971) and for induction of labor (Beazley, 1971; Ulstein et al., 1979; Squires and Masson, 1980; Lichtenegger et al., 1981). Intravenous and intrauterine administration of PGE_2 was shown to be equally effective when compared to $PGF_2\alpha$ in terminating pregnancies in cows during the last trimester of pregnancy (Zerobin et al., 1973). Kimball and Lauderdale (1975) found that a 25 mg im injection of PGE_1 did not affect plasma progesterone levels when administered to heifers on d 11 to 13 of the estrous cycle. On the other hand, Elger et al. (1981) found an orally administered PGE_1 analogue (CP 48630) to be effective in terminating pregnancy on d 43 and 44 of pregnancy in guinea pigs.

Thus, due to the known luteolytic and abortifacient capabilities of PGE_1, PGE_2 and $PGF_2\alpha$ and evidence for their presence in ponderosa pine needles, the possibility for their involvement in pine needle abortion exists. Even though the luteolytic and abortifacient abilities of these prostaglandins have been extensively reviewed, the mechanism by which they act is still not completely known. The possible mode of action for these luteolytic prostaglandins will be reviewed in the next section.

Theoretical Mode of Action for Luteolytic Prostaglandins

It was initially proposed that $PGF_2\alpha$ induced luteolysis by decreasing the blood flow to the ovary containing the corpus luteum (Pharris, 1970). This mode of action has been ruled out since reduction in arterial blood flow to the ovary-bearing corpora lutea is preceded by

a decline in serum progesterone by 6 h in rabbits (Bruce and Hillier, 1974) and 18 h in ewes (Nett et al., 1976).

Antagonism between $PGF_2\alpha$ and luteinizing hormone (LH) was suggested to be one of the mechanisms in $PGF_2\alpha$ luteolysis (Behrman et al., 1974). Consistent with this proposed mode of action, Hichens et al. (1974) showed that $PGF_2\alpha$ treatment reduced the number of receptors for human chorionic gonadotropin (HCG) in rat corpora lutea. It has been shown (Rao et al., 1976) that bovine corpora lutea contain $PGF_2\alpha$ receptors throughout pregnancy. These receptors had similar affinity for $PGF_2\alpha$ throughout pregnancy, but the number of available receptors varied. Similar receptors for PGE_1 (Rao, 1973; Kimball and Lauderdale, 1975) and PGE_2 (Rao, 1973) have been identified.

More recently, it has been reported that $PGF_2\alpha$ acts directly on luteal cells in vitro, markedly reducing LH-stimulated cyclic adenosine monophosphate (cAMP) accumulation (Dorflinger and Berman, 1980). Moreover, basal adenylate cyclase activity significantly drops along with serum progesterone within 6 h after $PGF_2\alpha$ injection in cattle (Fitz et al., 1980). This proposed mode of action for the luteolytic effect of $PGF_2\alpha$ is strengthened by the discovery (Spicer et al., 1981) that progesterone concentrations in cows injected with $PGF_2\alpha$ dropped 12 h earlier than changes in binding capacity of LH in corpora lutea. It was therefore speculated by these authors that the early antagonism of LH action on corpora lutea by $PGF_2\alpha$ was due to direct inhibition on adenylate cyclase to the LH receptor and not due to a decrease in numbers of LH binding sites during regression as observed by Hichens et al. (1974).

However, the reduction in LH receptors following $PGF_{2\alpha}$ administration may be a mechanism to insure that luteolysis continues once initiated.

Although there is presently no clinical diagnosis to positively identify pine needle abortion, similarities between the luteolytic and abortifacient capabilities of exogenous prostaglandin administration to cattle and the review by Stevenson et al. (1972) describing field observations of pine needle abortions suggest the involvement of a luteolytic agent such as prostaglandins. Some of these characteristics which implicate prostaglandins include (1) abortions can begin within 48 h after pine needle ingestion, indicating a rapid mode of action of the active agent, perhaps at the ovarian level (Liehr et al., 1972; Louis et al., 1972; Inskeep, 1973); (2) no observable signs of pending abortion following consumption of pine needles with the abortion being characterized by weak parturition contractions (Zerobin et al., 1973); (3) calves born rapidly or rescued from the uterus early during parturition are as vigorous and healthy as their stage of development will allow (Henricks et al., 1977); (4) pine needle abortion is characterized by a persistent retained placenta regardless of the gestational stage of the abortion (Zerobin et al., 1973; Henricks et al., 1977; Copeland et al., 1978; Plenderleith, 1978); (5) a purulent discharge and palpable metritis are common characteristics associated with pine needle abortion (Copeland et al., 1978; Sequin et al., 1978) and (6) some cows may show signs of estrus very shortly after they abort (Zerobin et al., 1973).

Toner (1971) observed a significant increase ($P<.05$) in plasma estrogen levels with steadily declining progesterone levels following

oral administration of a pine needle drench to pregnant heifers during a 3-d treatment period. This fluctuation in estrogen and progesterone levels was similar to those observed following administration of a luteolytic dose of $PGF_{2\alpha}$ to cattle (Louis et al., 1974; Chenault et al., 1976).

It is known that pregnancy is maintained in sheep during the first 55 d by the action of progesterone produced by the corpus luteum (Casida and Warwick, 1945; Foote et al., 1957). Thereafter, the placenta synthesizes sufficient progesterone to maintain pregnancy. Call and James (1976) conducted an experiment in which they concluded that consumption of pine needles by sheep did not interfere with pregnancy. Pregnancy and lambing rates were not affected in 30 mature ewes by the feeding of pine needles during d 60 to 90 of gestation and there were no signs of toxicity. However, pine needles that were collected in the same area subsequently induced abortion in cattle (James et al., 1977). In contrast to the ewe, the cow relies on an ovarian source of progesterone up to an ill-defined time between d 150 to 250 of gestation (Barth et al., 1981). Thus, the different results obtained following feeding of pine needles between these two species suggest a luteolytic mode of action of the active pine needle factor(s).

Although luteolytic agents seem to be causative agents in pine needle abortion, this malady has yet to be adequately differentiated from other abortive diseases in cattle. One reason for the incomplete characterization of pine needle abortion is the unpredictable nature of the problem (Olson, 1976). Undefined causative factors involved in pine

needle abortion other than those attributed to actual pine needle constituents may also play an important role in this disorder. In a review of pine needle abortion by Call and James (1978), undefined causative factors in pine needle abortion such as environmental stress, body condition, disease and biological differences may influence the effect of pine needles in the individual animal. Several factors apparently cause cattle to eat pine needles, which in turn may predispose animals to the abortifacient agents contained within them. These include (1) sudden weather changes, such as cold winds or snow storms when cattle seek shelter in trees and eat the needles; (2) starvation when feed becomes scarce near the end of the grazing season before cattle are moved from the range; (3) changes in feed, especially to unfamiliar or poor quality feed; (4) sudden access to pine needles, especially when cattle are moved from the pine-free fields or pastures; (5) boredom or (6) accidental ingestion with other food.

Due to the possibility of these and other predisposing factors involved in pine needle abortion, research involving experimental feeding of ponderosa pine needles has not consistently resulted in abortions. Until the effects of the nonpine needle environmental parameters can be understood, the actual amounts of individual pine needle constituents required to cause abortion will be difficult to determine. Accordingly, preventive measures other than present managerial practices will probably not be developed until we possess a comprehensive understanding of direct and indirect factors involved in pine needle abortion.

MATERIALS AND METHODS

Dose-Response Experiments With Mice

The objectives of these experiments were to investigate the effects of known prostaglandins and a diterpene resin acid on pregnant mice and to establish dose-response data during late gestation. In Exp. A and B, $PGF_2\alpha$ was administered ip to 12-d pregnant mice. In Exp. C and D, $PGF_2\alpha$ was administered ip to 10-d pregnant mice. In Exp. E, 12-d pregnant mice were treated with oral administrations of $PGF_2\alpha$, PGE_2 and abietic acid.

Random bred mature female mice (Mus musculus) derived from the HA/ICR strain and inbred mice (129/Sv and C57BL/6J strains) were used in these experiments. Female mice were placed one or two per cage with males and examined daily throughout a 5-d breeding period for the presence of a copulatory plug. The day a copulatory plug was observed was designated as d 0 of pregnancy. Pregnancy was verified by noting a substantial weight increase at the time of treatment compared to d 0 of pregnancy. Pregnant mice were kept in plastic cages with straight wire lids and maintained under controlled environmental conditions (16 h light-8 h dark, temperature 22 C, 50% relative humidity) with Wayne Lab-Blox and water provided ad libitum throughout the experiment.

At the termination of the treatment period, all mice were weighed and then killed by cervical dislocation. Three parameters were measured: (1) each uterus was removed by cutting at the uterotubal junction and cervix with each fetus contained within the uterine horns scored as alive or resorbed; (2) viable and nonviable fetuses plus placentas were

removed from the uterine horns and weighed on a Mettler precision spring balance, recorded as total fetal weight per mouse and (3) ovaries were removed from the ovarian bursa with the aid of a dissecting microscope and the total number of corpora lutea and total ovarian weight were recorded.

Experiment A. Sixty female mice (HA/ICR and 129/Sv) were exposed to males (HA/ICR) during a 5-d breeding period. Of the 35 mice showing copulatory plugs at the termination of this period, 33 became pregnant. In this experiment and in Exp. B, C and D, $PGF_2\alpha$[1] was serially diluted in .2M phosphate buffer (pH 7.4) to concentrations of 300, 150, 75 and 37.5 µg/.1 ml delivered volume. Concentrations of $PGF_2\alpha$ used in these dose-response experiments were based on mouse experiments conducted by Persuad (1974), who showed that 100 µg $PGF_2\alpha$ injected sc daily from d 12 to 15 of gestation terminated pregnancy. Pregnant mice received one ip injection of $PGF_2\alpha$ on d 12 of gestation with control mice receiving a similar volume of .2M phosphate buffer (pH 7.4). All mice were killed and examined on d 17 of the 21-d gestation period (table 1).

Experiment B. Fifty female mice (HA/ICR and C57BL/6J) were exposed to males (HA/ICR) during a 5-d breeding period. Of the 48

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[1] $PGF_2\alpha$ used in these experiments was Lutalyse marketed by the Upjohn Pharmeceutical Company, Kalamazoo, MI. Lutalyse contains the naturally occurring $PGF_2\alpha$, dinoprost, as the tromethamine salt. Each ml of Lutalyse contains dinoprost tromethamine equivalent to 5 mg of dinoprost.

TABLE 1. MICE DOSE-RESPONSE EXPERIMENTS

Experiment (treatment)	Route of administration	Dosage, µg/treatment	No. of treatments	No. of mice	Day of gestation[a] At treatment	Day of gestation[a] At examination
A ($PGF_2\alpha$)	ip	300	1	5	12	17
	ip	150	1	4	12	17
	ip	75	1	4	12	17
	ip	37.5	1	4	12	17
	ip	0	1	6	12	17
B ($PGF_2\alpha$)	ip	300	1	7	12	17
	ip	150	1	8	12	17
	ip	75	1	8	12	17
	ip	37.5	1	8	12	17
	ip	0	1	7	12	17
ovariectomy				6	12	17
C ($PGF_2\alpha$)	ip	300	1	3	10	15
	ip	150	1	4	10	15
	ip	75	1	1	10	15
	ip	37.5	1	2	10	15
	ip	0	1	3	10	15
D ($PGF_2\alpha$)	ip	300	1	4	10	12,13,14
	ip	150	1	3	10	11,12,14
	ip	75	1	3	10	11,12,13
	ip	37.5	1	5	10	11,12,13,14
	ip	0	1	3	10	11,12,13
E						
Part 1 ($PGF_2\alpha$)	oral	1500	1	17	12	17
	oral	750	1	17	12	17
Part 2 (PGE_2)	oral	1000	1	5	12	17
	oral	500	1	5	12	17
	oral	0	1	5	12	17
Part 3 (abietic acid)	oral	5×10^4	1	6	12	17
	oral	2.24×10^4	1	8	12	17
	oral	1×10^4	1	8	12	17
	oral	0	1	3	12	17

[a] Based on a 21-d gestation period.

mice showing copulatory plugs at the termination of this period, 44 became pregnant. $PGF_2\alpha$ concentrations and injection schedule employed in Exp. A were replicated (table 1). In addition to the mice receiving $PGF_2\alpha$ treatments, six pregnant mice were used to confirm the absolute necessity of corpus luteum in maintaining pregnancy in the mouse (Murr et al., 1974; Pointis et al., 1981). Bilateral ovariectomies were performed on three pregnant mice on d 12 of gestation. Three 12-d pregnant mice served as controls and underwent identical surgery without removal of the ovaries.

Experiment C. Forty female mice (HA/ICR, 129/Sv and C57BL/6J) were exposed to males (HA/ICR) during a 5-d breeding period. Of the 33 mice showing copulatory plugs at the termination of this period, 13 became pregnant. $PGF_2\alpha$ treatments were injected ip on d 10 of gestation and all mice killed and examined on d 15 (table 1).

Experiment D. Eighteen pregnant female mice received a single ip injection of $PGF_2\alpha$ on d 10 of gestation and were killed and examined on either d 11, 12, 13 or 14 in order to determine at what point following $PGF_2\alpha$ administration embryonic mortality occurred.

Experiment E. One hundred eighteen female mice (HA/ICR) were exposed to males (HA/ICR) during a 5-d breeding period. Of the 101 mice showing copulatory plugs at the termination of this period, 74 became pregnant. As indicated in table 1, Exp. E consisted of three separate treatments: (1) $PGF_2\alpha$ administered orally via stomach

tube[2] on d 12 of gestation, (2) PGE_2[3] administered orally via stomach tube on d 12 of gestation and (3) abietic acid[4] administered orally via stomach tube on d 12 of gestation. $PGF_2\alpha$ was diluted in .2M phosphate buffer (pH 7.4) to concentrations of 1500 µg/.3 ml and 750 µg/.2 ml delivered volume. These concentrations were based on those used by Goyings (1979) who reported a decrease in mean number of fetuses per litter with oral administration of $PGF_2\alpha$ at 600 µg/30-g rat. A stock solution of PGE_2 at $1x10^4$ µg/ml was diluted in .2M phosphate buffer (pH 7.4) to concentrations of 1000 and 500 µg/.2 ml delivered volume. Concentrations of abietic acid used in this study were based on those obtained by Kubik and Jackson (1981) who found that 22.4 mg/d of a diterpene resin acid mixture terminated pregnancy in mice when administered orally during d 1 to 5 of gestation. Abietic acid was diluted in ethanol to concentrations of $5x10^4$, $2.24x10^4$ and $1x10^4$ µg/.2 ml delivered volume. All mice were killed and examined on d 17 of gestation.

Mice Experiments Testing Pine Needle Fractions For Prostaglandin Activity

The procedure to fractionate, isolate and purify prostaglandins from plant material has been reported by Attrep et al. (1979). The

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[2]The stomach tube apparatus used in the administration of these treatments consisted of a 4 cm x 1 mm Clay-Adams Intermedic polyethylene tube connected to a Tuberculin syringe with a 25G x 16 mm needle.

[3]PGE_2 was obtained from Sigma Chemical Company, St. Louis, MO, Product No. 5640.

[4]Abietic acid was obtained from Sigma Chemical Company, St. Louis, MO, Product No. A-0125.

procedure for the separation and isolation of prostaglandins from ponderosa pine needles employed by Attrep is outlined in figure 1.

The biological activity of three separate fractions from this purification scheme were tested utilizing pregnant mice. As indicated in figure 1, these fractions corresponded to (1) crude pine needle extract, (2) extract before column separation and (3) fractions collected following column separation representing individual prostaglandin-containing series.

The ponderosa pine needles used in this study were collected from the Billy Lei Ranch at Brownsville, South Dakota. Needles were stripped from their branches, ground in a hammer mill to approximately 3-cm lengths and then stored in plastic bags at -10 C until used.

Experiment 1, Fraction 1. In this experiment, pine needle extracts of 0, 25, 50 and 100 g of pine needles were tested for biological activity. The volume of .2M phosphate buffer (pH 7.4) used in the extraction process remained constant at 250 ml, giving equivalent pine needle concentrations of 0, $1x10^4$, $2x10^4$ and $4x10^4$ µg pine needle extract per .1 ml buffer (table 2). Pine needles were homogenized in a cold room (4 C) using a commercial Waring blender. Pine needle homogenate was strained through cheesecloth (20x12 threads/2.54 cm^2) with retained fiber (pulp) discarded. Filtered extract was stored in vials at 4 C until used. Fifty pregnant mice (C57Bl/6J, 129/Sv and HA/ICR) were used in testing the biological activity of these buffer extracts. Extracts were administered orally during a 5-d treatment period

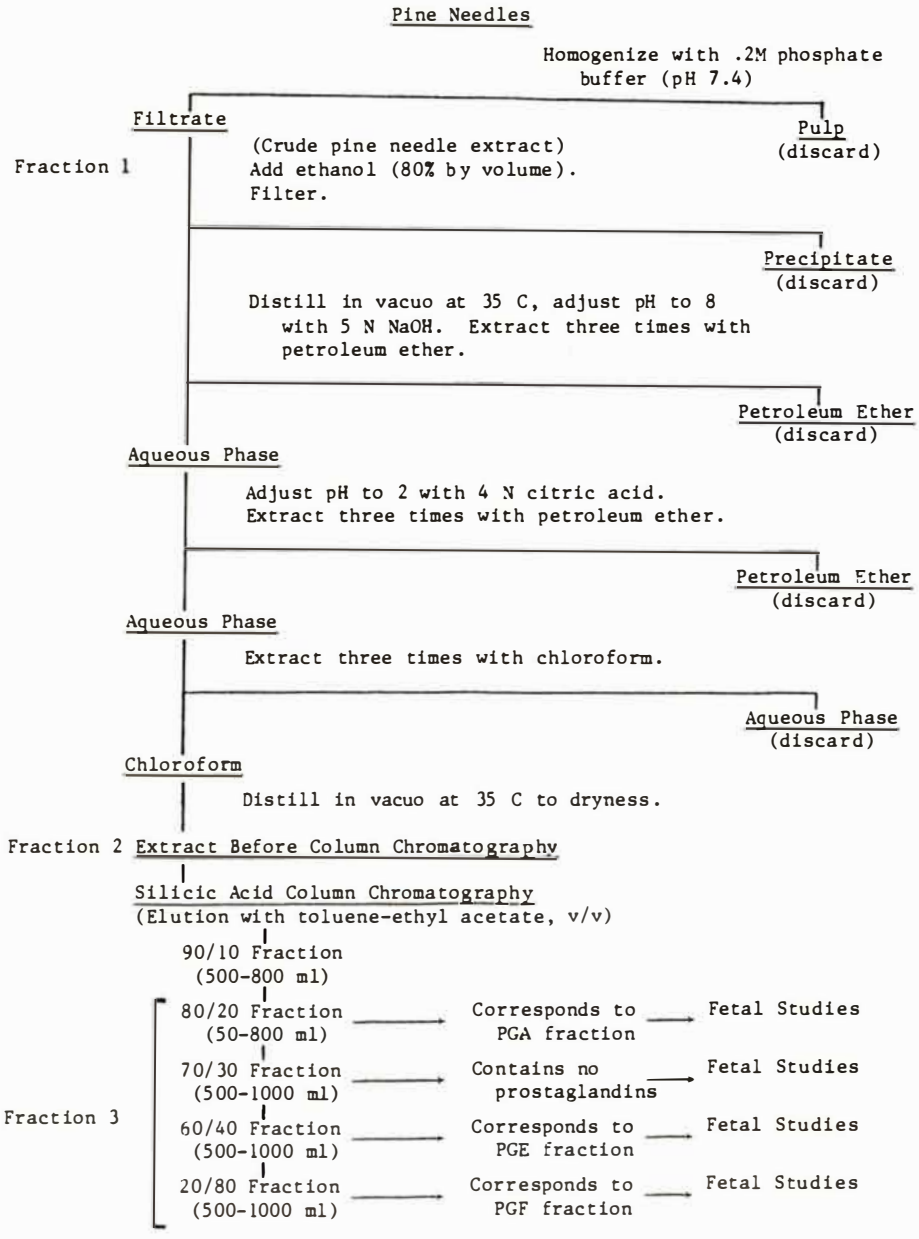

Figure 1. Procedure used by Moses Attrep for the isolation of fatty acid-prostaglandin fractions from pine needles.

TABLE 2. MICE PINE NEEDLE FRACTION EXPERIMENTS

Experiment (treatment)	Route of administration	Dosage, µg/treatment	No. of treatments	No. of mice	Day of gestation[a] At treatment	Day of gestation[a] At examination
1 (Fraction 1)	Oral	$8x10^4$	5	14	12-16	17
	Oral	$4x10^4$	5	14	12-16	17
	Oral	$2x10^4$	5	13	12-16	17
	Oral	0	5	9	12-16	17
2 (Fraction 1)	Oral	$16x10^4$	5	4	12-16	17
	Oral	$12x10^4$	5	5	12-16	17
	Oral	$8x10^4$	5	4	12-16	17
	Oral	0	5	2	12-16	17
1 (Fraction 2)	ip	3378	1	11	12	17
	ip	1689	1	8	12	17
	ip	844.5	1	4	12	17
	ip	422.25	1	7	12	17
	ip	0	1	8	12	17
2 (Fraction 2)	Oral	4140	1	7	10	15
	Oral	2070	1	3	10	15
	Oral	1076	1	4	10	15
	Oral	0	1	5	10	15
3	ip	1715	3	1	10-12	15
	ip	1715	2	5	10-11	15
	ip	1715	1	3	10	15
	ip	0	3	3	10-12	15
1 (Fraction 3)						
PGA	Oral	17070	2	6	12-13	17
PGE	Oral	74800	2	5	12-13	17
PGF	Oral	58830	2	6	12-13	17
70/30	Oral	213790	2	4	12-13	17

[a] Based on a 21-d gestation period.

beginning on d 12 of gestation at a delivered volume of .2 ml/treatment. Control animals received a similar volume of buffer. All animals were killed and examined on d 17 of gestation.

Experiment 2, Fraction 1. The procedure used in Exp. 1 was duplicated in Exp. 2. Fifteen pregnant mice received pine needle extracts at concentrations representing 0, $4x10^4$, $6x10^4$ and $8x10^4$ µg pine needle extract per .1 ml buffer.

Fraction 2[5], indicated as extract before column separation (figure 1), was obtained following further purification of fraction 1. After separation of the pine needle fiber, the protein fraction of the crude pine needle extract was denatured by adding enough ethanol to make the solution 80% ethanol by volume. This solution was allowed to stand in a 3 C environment for at least 15 h and then centrifuged to remove the denatured protein. The ethanol was then removed by vacuum distillation at 35 C. The remaining aqueous phase was adjusted to pH 8 with 5 N NaOH and extracted three times with petroleum ether (1 ml petroleum ether:1 ml aqueous phase). The aqueous phase was then adjusted to pH 2 with 4 N citric acid and extracted three times as before with petroleum ether. The next phase of the purification process was the extraction of the aqueous phase with three volumes of chloroform (1:1 v/v). The combined chloroform extracts were distilled in vacuo at 35 C to dryness. The small amount of oily residue remaining in the flask was

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[5] Fractions 2 and 3 were obtained from Dr. Moses Attrep and Katherine Attrep, East Texas State University, Commerce, TX.

removed with 3:2-ml portions of ethanol. The ethanol was evaporated under a stream of N_2.

The oil fraction isolated at this stage of the purification process probably represented a wide variety of organic components. Particular emphasis was placed on testing the lipid component, since prostaglandins or their precursors should be contained in this fraction. Considerable difficulty was encountered in solubilizing this fraction which showed limited solubility in nontoxic solvents. Three separate experiments were conducted in testing the biological activity of fraction 2: (1) ip injections of a dimethyl sulfoxide (DMSO)-soluble fraction, (2) DMSO-soluble fraction administered orally and (3) ip injections of a buffer-soluble fraction.

Experiment 1, Fraction 2. In this experiment, 168.9 mg of fraction 2 was serially diluted in 5 ml of DMSO corresponding to concentrations of 0, 422.25, 844.5, 1689 and 3378 µg/.1 ml DMSO (table 2). Fifty-eight female mice (HA/ICR and 129/Sv) were exposed to males (HA/ICR) during a 5-d breeding period. Of the 46 mice showing copulatory plugs at the termination of this period, 38 became pregnant. Pregnant mice received one ip injection on d 12 of gestation with control mice receiving a similar volume of DMSO. All mice were killed and examined on d 17.

Experiments 2 and 3, Fraction 2. Fraction 2 was separated into buffer-soluble and nonbuffer-soluble subfractions to individually test their biological activity. Phosphate buffer (2 ml) was added to 81.5 mg

of fraction 2. After mixing and separation, 34.3 mg of the 81.5-mg sample was found to be soluble in buffer, giving a concentration of 1715 µg/.1 ml buffer. The remaining nonbuffer-soluble fraction was diluted in DMSO to concentrations of 0, 1076, 2070 and 4140 µg/.1 ml DMSO (table 2). Pregnant mice received one administration via stomach tube of DMSO-soluble fraction 2 on d 10 of gestation with corresponding controls receiving a similar volume of DMSO. Buffer-soluble fraction 2 was injected ip over a 1-, 2- or 3-d treatment period beginning on d 10 of gestation. All mice were killed and examined on d 15 of gestation.

Experiment 1, Fraction 3. Fraction 3 (figure 1) represents four individual fractions collected following column chromatographic separation of fraction 2. Sil-A-200 silicic acid prepared for resolution of acidic mixtures with a mesh size of 60-200 was used in this silicic acid column chromatographic separation procedure. Silicic acid (7.89 g) was combined with 25 ml of the eluting solvent (toluene-ethyl acetate; 9:1 v/v). This slurry was poured into a column 1 cm in diameter and 50 cm in length. A purified pine needle extract (fraction 2) weighing 70 to 100 mg was dissolved in the toluene-ethyl acetate (9:1) and introduced on the column head. Four separate fractions were collected following column separation corresponding to the prostaglandin A series, prostaglandin E series, prostaglandin F series and a fraction containing no prostaglandins. Individual column fractions were collected in preweighed plastic vials such that weights of individual fractions could be determined. Excess solvent was evaporated under a stream of N_2, with the remaining sample diluted in 1 ml of DMSO to concentrations indicated

in table 2. Twenty-one pregnant mice (HA/ICR) were utilized in testing the biological activity of these prostaglandin-containing fractions. Fractions were administered via stomach tube once daily at a delivered volume of .1 ml per treatment on d 12 and 13 of gestation. Control animals received similar volumes of sample 70/30 of fraction 3 (figure 1) which was not believed to contain prostaglandins. All mice were killed and examined on d 17 of gestation.

Statistical Analyses of Mice Experiments. Fetal viability data were tested using Chi-square analysis. Independent Chi-square values were determined to identify significant treatment effects compared to controls. Statistical analyses of viable fetal weight, resorbed fetal weight and ovarian weight were conducted based on the Statistical Analysis System (SAS) using the least-squares analysis of variance to examine treatment effects. Mouse body weight at d 0 of pregnancy was used as a covariate in analyzing viable fetal weight and resorbed fetal weight. Differences between treatment means were compared by using the Waller-Duncan K-ratio test (Steel and Torrie, 1980). The analyses of variance for all variables in the mice experiments are presented in appendix tables 1 through 22.

Cattle Pine Needle Feeding Experiment

The limited number of experimental animals employed in this part of the study allowed only descriptions of distinct changes following pine needle consumption and these experiments were intended to serve as a pilot study for future research. The objectives were (1) to produce experimental abortions in cattle by feeding ponderosa pine needle diets, (2) document

gross pathological changes in maternal and fetal systems following abortions and (3) to monitor blood serum progesterone levels along with total and differential leukocyte numbers to establish preliminary data on mode of action for causative agents in pine needle abortion.

Eight pregnant Angus cows were used in the experiment and ranged from 367 to 434 kg in weight. All animals were determined to be in at least the fifth month of gestation by rectal palpation. A period of 12 d (January 6 to January 17, 1983) was used for adjustment following transport of cattle from Naper, Nebraska. During this period, all cattle were housed in outdoor pens at the South Dakota State University Beef Cattle and Nutrition Unit and fed prairie hay (87% dry matter) with free access to water. Proximate analyses of prairie hay and ponderosa pine needles fed in this experiment are shown in tables 3 and 4, respectively.

Because undefined environmental stress factors may predispose animals to pine needle consumption and ultimately abortions, cattle were divided into two experimental groups. Following the 12-d adjustment period, four cows were moved indoors to individual pens in the large animal facility at the Animal Science Complex and four cows remained outdoors at the nutrition unit.

Indoor Pine Needle Feeding Experiment. Two cows received pine needle diets and the remaining two cows served as controls (table 5). One control animal was fed an adequate amount of prairie hay (based on 4 kg TDN daily requirement; NRC, 1976) and one received prairie hay at a level corresponding to the intake of animals fed pine needle diets.

TABLE 3. PROXIMATE ANALYSIS PARAMETERS OF PRAIRIE HAY

Item	Dry matter basis, %	As fed basis, %
Moisture	0	12.7
Crude protein	5.5	4.8
Crude fiber	36.4	31.8
Ether extract	2.78	2.43
Ash	9.54	8.33
Nitrogen-free-extract	45.8	40.0

TABLE 4. PROXIMATE ANALYSIS PARAMETERS OF PONDEROSA PINE NEEDLES

Item	Dry matter basis, %	As fed basis, %
Moisture	0	39.5
Crude protein	6.54	3.96
Crude fiber	25.20	15.3
Ether extract	9.67	5.85
Ash	1.80	1.09
Nitrogen-free-extract	56.7	34.3

TABLE 5. INDOOR PINE NEEDLE FEEDING SCHEDULE[a]

Cow ID no.	Treatment period, d	Pine needles in treatment/day, kg	Prairie hay fed/day, kg
377	1 to 4[b]	4.5	--
	5 to 7[c]	2	2.5
	11 to 15 and 17[d]	2,2.5,2,3,2.5,3	4.5
	28 to 32[e]	6.8,6.8,3.4,3.4,3.4	6
374	1 to 4[b]	4.5	--
	5 to 7[c]	2	2.5
	11 to 15 and 17[d]	3,2.5,3,3,2.5,3	4.5
	28 to 32[e]	6.8,6.8,3.4,3.4,3.4	6
373	1 to 4	--	9
	5 to 7	--	9
	11 to 15 and 17[f]	--	9
	28 to 32[f]	--	9
379	1 to 4	--	4.5
	5 to 7	--	4.5
	11 to 15 and 17[f]	--	4.5
	28 to 32[f]	--	6

[a] All animals were fed 6.8 kg prairie hay per day for 2 d prior to the 32-d treatment period.

[b] Starting with first day of treatment period, corresponding to January 19, 1983. Pine needle treatment consisted of chopped pine needles.

[c] Treatment consisted of chopped pine needles mixed with prairie hay.

[d] Treatment consisted of pine needle extract administered via stomach tube.

[e] Treatment consisted of pine needle pulp mixed with phosphate buffer and administered via stomach tube.

[f] Control animals no. 373 and 374 received a similar volume of phosphate buffer as animals receiving pine needle treatments.

Cows receiving pine needle diets were first offered 4.5 kg chopped green pine needles for a 4-d period but refused to eat the needles. A pine needle-prairie hay mixture (1:2) was then offered for a 3-d period but was also refused. Following refusal of these pine needles diets, pine needle extracts were prepared and force fed via stomach tube. Pine needles were extracted with .2M phosphate buffer (pH 7.4) as previously described for the mice experiments (experiment 1, fraction 1) with the total amount of pine needles extracted per volume of buffer administered indicated in table 5. Pine needle pulp remaining after extract preparation was refrigerated for later use.

Cattle received pine needle extracts once daily (AM) for a 6-d treatment period, and control animals received a similar volume of phosphate buffer. Prior to each treatment, all cattle were weighed and blood samples collected via jugular venipuncture. Nonheparinized blood samples were collected for serum progesterone assays. Heparinized samples were used in leukocyte counts. Cattle receiving pine needle extracts were also fed 4.5 kg prairie hay daily.

Following pine needle extract treatment, cattle were observed during an 11-d period for signs of toxicity and pending abortion. Since no effects of these treatments were observed, a pine needle pulp-phosphate buffer slurry was prepared and administered orally for a 5-d period (table 5). Pine needle pulp remaining after extract preparation for the previous treatment was mixed with phosphate buffer to produce a slurry which would flow through a stomach tube. Treatments were administered twice daily (AM and PM) for a 2-d period and then only once daily (AM)

for a 3-d period due to problems with regurgitation at the PM treatment. Control animals received a similar volume of phosphate buffer during this 5-d period. All animals were weighed and bled daily prior to treatment. Cattle receiving pine needle pulp were also fed 6 kg prairie hay daily.

Outdoor Pine Needle Feeding Experiment. The four cattle used in this pine needle feeding experiment were divided into two groups corresponding to cattle receiving pine needle diets and those receiving prairie hay (table 6). Prior to feeding pine needle diets (January 6 to February 7, 1983), all cattle were fed prairie hay. Ground pine needles were then phased into prairie hay diets over a 26-d feeding period (February 8 to March 5, 1983) beginning at 10% of the diet and terminating at 35% of the diet. Control animals were fed prairie hay during this 26-d period. The amounts fed corresponded to the level of intake of animals receiving pine needle diets. All animals were periodically weighed and bled throughout the study.

Blood Sample Analysis

Leukocyte counts from heparinized blood samples were made by South Dakota State University Animal Disease Research Diagnostic Laboratory personnel. Serum samples were analyzed by radioimmunoassay for progesterone concentrations according to the procedures of Maurer and Echternkamp (1982). Sample recovery of extractions of spiked steer serum (6 ng/ml) ranged from 96 to 99% with an average progesterone recovery of 6.89 ng/ml. Percent binding ranged from 35 to 50% with a

TABLE 6. OUTDOOR PINE NEEDLE FEEDING SCHEDULE

Treatment, period, d	Cow ID no. 372 and 378 Treatment: Pine needles, kg/ prairie hay, kg		Cow ID no. 375 and 376 Treatment: Prairie hay, kg[b]
1 to 11	0/12	(0)[a]	12
12 to 32	0/16	(0)	16
33[c]	4/36	(10)	40
34 to 35	8/32	(20)	40
36 to 37	2/18	(10)	20
38	3/17	(15)	20
39 to 40	4.5/25.5	(15)	30
41 to 42	6/24	(20)	30
43 to 47	7/23	(23)	30
48	9/21	(30)	30
49 to 52	12/28	(30)	40
53 to 58	14/26	(35)	40

[a] Numbers in parentheses represent total percentage of pine needles in diet for two experimental animals fed together.
[b] Total kilograms prairie hay for two control animals fed together.
[c] Starts first day of 26-d pine needle feeding period, corresponding to February 8, 1983.

background between 2 and 3%. The standard curve had a correlation coefficient of .9990, slope of -.8799 and intercept of 3.82. Varying volumes of steer serum were assayed against standards and found to be parallel. The intraassay coefficient of variation based on four spiked steer serum samples was 13% and the interassay coefficient of variation based on 20 samples was 8.72%.

RESULTS AND DISCUSSION

Dose Response Experiments With Mice

Experiment A. The objective of this experiment was to establish dose-response data on the effects of $PGF_2\alpha$ on pregnant mice. Pregnant mice injected ip with 300, 150, 75 or 37.5 µg $PGF_2\alpha$ on d 12 of gestation had a significantly higher ($P<.01$) incidence of fetal deaths and resorptions compared to controls receiving a similar volume of phosphate buffer (table 7). The highest percentage of fetal resorptions occurred at the 300 µg/.1 ml concentration. Fetal viability increased with decreasing concentrations of $PGF_2\alpha$. Viable fetuses and corresponding placentas examined on d 17 of gestation showed no apparent malformations and appeared normal in appearance when compared to control fetuses of a similar stage of gestation. Uteri of mice containing resorbed fetuses were involuted and characteristic fetal form was absent (figure 2). Placentas were also in a state of resorption, although not to the degree

TABLE 7. CHI-SQUARE ANALYSIS OF $PGF_2\alpha$ TREATMENT ON FETAL VIABILITY COMPARED TO CONTROLS (EXPERIMENT A)

Treatment	No. viable fetuses	No. dead fetuses	χ^2
$PGF_2\alpha$, µg/.1 ml			
300	4 (7.1)[a]	52 (92.9)	107.8172**
150	25 (42.4)	34 (57.6)	52.8830**
75	33 (61.1)	21 (38.9)	31.5385**
37.5	32 (66.7)	16 (33.3)	25.9315**
Control	67 (100)	0 (0)	

[a] Numbers within parentheses represent percentages.
** $P<.01$, 1 d.f.

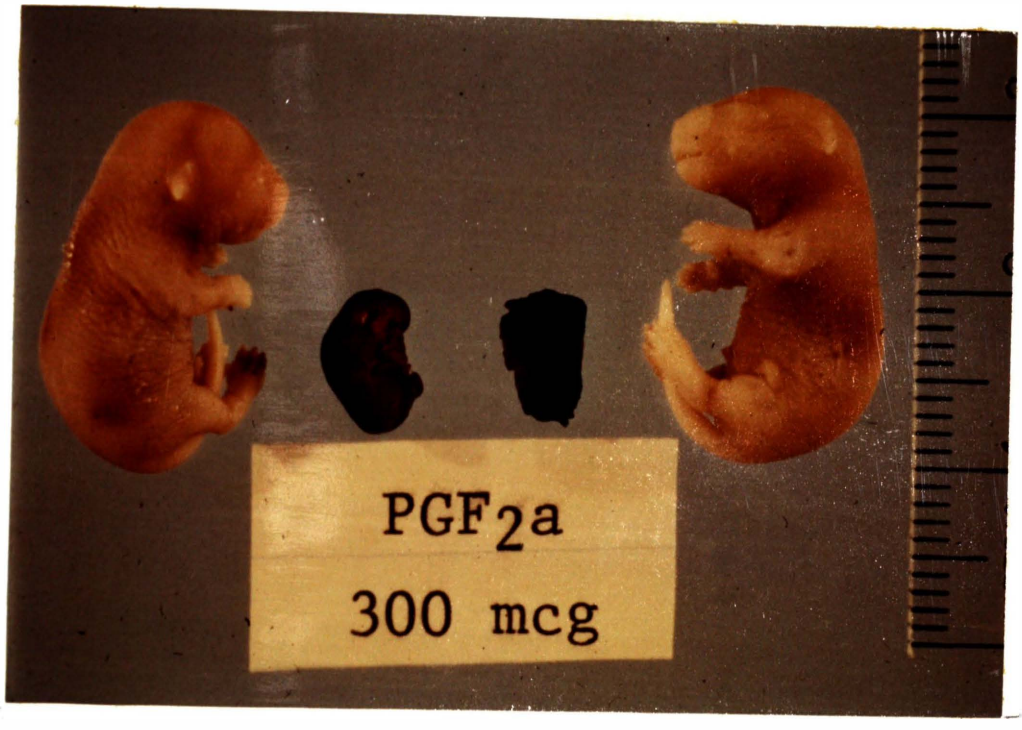

Figure 2. Resorbed fetuses compared to controls at d 17 of gestation following ip injection of 300 μg $PGF_2\alpha$.

of the corresponding resorbed fetuses. Ovaries of treated mice showed no morphological changes indicative of damage other than the presence of corpora albicans corresponding to resorbed fetuses. Although a count of corpora lutea is more indicative of the number of ova ovulated rather than number of implantations or number of fetuses contained within the uterus (Rugh, 1968), it appeared from this experiment that the number of corpora lutea closely approximated the number of viable fetuses, with corpora lutea of resorbed fetuses apparently degenerating within the 5-day treatment period. This study served to confirm early investigations (Bartke et al., 1971; Labhsetwar, 1972; Marley, 1972; Persuad, 1974) showing the luteolytic capabilities of $PGF_2\alpha$ in terminating pregnancy in the laboratory mouse. There did not appear to be a specific

uterine pattern to fetal resorptions, because resorbed fetuses were equally observed in both uterine horns and in intermittent positions among viable fetuses. The effects of $PGF_2\alpha$ on fetal viability appeared to be all or none, since fetuses were either resorbed or normal in appearance upon examination on d 17 of gestation.

Experiment B. $PGF_2\alpha$ concentrations and injection schedule employed in Exp. A were replicated. Mice receiving ip injections of 300, 150 and 75 µg $PGF_2\alpha$ had a significantly higher ($P<.01$) percentage of fetal resorptions upon examination on d 17 of gestation than mice administered phosphate buffer (table 8). Administration of 37.5 µg $PGF_2\alpha$ resulted in a higher percentage of fetal resorptions than controls. However, this difference was not significant. Fetuses not affected by $PGF_2\alpha$ showed no apparent malformations and appeared normal. Individual viable and resorbed fetuses along with placentas were removed from the

TABLE 8. CHI-SQUARE ANALYSIS OF $PGF_2\alpha$ TREATMENT ON FETAL VIABILITY COMPARED TO CONTROLS (EXPERIMENT B)

Treatment	No. viable fetuses	No. dead fetuses	χ^2
$PGF_2\alpha$, µg/.1 ml			
300	2 (3.5)[a]	55 (96.5)	55.8768**
150	23 (24.5)	71 (75.5)	30.5375**
75	26 (29.5)	62 (70.5)	24.0742**
37.5	65 (75.6)	21 (24.4)	.5194
Ovariectomized (Sham)	79 (94.0)	5 (6.0)	3.6601
Control	23 (82.1)	5 (17.9)	

[a] Numbers within parentheses represent percentages.
** $P<.01$, 1 d.f.

uterus and weighed to determine differences in fetal weights between treatments (table 9). Average viable fetal weights of mice receiving ip injections of 300 µg $PGF_2\alpha$ were significantly lower ($P<.05$) than average fetal weights from mice receiving 75 µg $PGF_2\alpha$, 37.5 µg $PGF_2\alpha$ and control treatments. Controls receiving phosphate buffer had significantly lower average resorbed fetal weights than mice receiving the 75 µg treatment. This difference in resorbed fetal weights observed within the control treatment may be due in part to fetuses being resorbed prior to treatment at d 12 of gestation.

The mouse ovary possesses marked cycles of ova maturation and hormone production. However, there is little change in ovarian weight except in pregnancy (Bronson et al., 1966). This increase in weight is due to excess fluid and cell production associated with corpora lutea formation. Assuming a 1:1 relationship of corpora lutea to viable fetuses, ovarian weight was expected to decrease with increasing concentration of $PGF_2\alpha$ and serve as a measure of fetal viability. Interestingly however, no differences in average total ovarian weight were noted between treatment groups (table 9).

In addition to obtaining dose-response data, the second objective of this experiment was to confirm the absolute necessity of the corpus luteum in maintaining pregnancy in the mouse. Bilateral ovariectomies performed on three pregnant mice on d 12 of gestation resulted in abortion of all fetuses within 48 h following surgery. Fetal viability was not affected in control mice which underwent identical surgery with the exception of ovary removal (table 8). The

TABLE 9. LEAST-SQUARES MEANS FOR VIABLE FETAL WEIGHTS, RESORBED FETAL WEIGHTS AND OVARIAN WEIGHTS AS AFFECTED BY $PGF_2\alpha$ TREATMENT (EXPERIMENT B)

Item	$PGF_2\alpha$, µg/.1 ml										SD[a]
	300		150		75		37.5		0		
No. of mice	7		8		8		8		7		
Avg viable fetal wt, g	.1860[c]	(2)[d]	.6860[bc]	(23)	.8168[b]	(26)	.8065[b]	(65)	.9664[b]	(23)	.4623
Avg resorbed fetal wt, g	.0656[bc]	(55)	.0657[bc]	(71)	.0823[b]	(62)	.0694[bc]	(21)	.0216[c]	(5)	.0405
Avg ovarian wt, g	.0122	(7)	.0107	(8)	.0115	(8)	.0128	(8)	.0128	(7)	.0043

[a] Standard deviation derived from pooled error term.
[b,c] Means within rows followed by unlike superscripts differ ($P<.05$).
[d] Values within parentheses indicate number of observations per mean.

fact that mice depend on an ovarian source of progesterone throughout pregnancy facilitates their use in testing the hypothesis of luteolytic agents in ponderosa pine needles.

Experiment C. In this experiment, pregnant mice received ip injections of $PGF_2\alpha$ on d 10 of gestation and were analyzed on d 15. Experiments by Bartke et al. (1972), Labhsetwar (1972) and Marley (1972) showed that $PGF_2\alpha$ administered to pregnant mice during early gestation (d 1 to 7) caused luteolysis and termination of pregnancy. Moreover, the concentration of $PGF_2\alpha$ required to terminate pregnancy during early gestation was less than that required to produce comparable results after midgestation (Persuad, 1974). As indicated in table 10, all $PGF_2\alpha$ treatments with the exception of the 37.5 µg concentration resulted in a higher ($P<.01$) incidence of fetal death than in control animals. Administration of 300 and 75 µg $PGF_2\alpha$ resulted in 100% fetal resorption.

TABLE 10. CHI-SQUARE ANALYSIS OF $PGF_2\alpha$ TREATMENT ON FETAL VIABILITY COMPARED TO CONTROLS (EXPERIMENT C)

Treatment	No. viable fetuses	No. dead fetuses	χ^2
$PGF_2\alpha$, µg/.1 ml			
300	0 (0)[a]	23 (100)	32.3704**
150	13 (35.1)	24 (64.9)	12.4376**
75	0 (0)	8 (100)	17.0458**
37.5	13 (65)	7 (35)	1.7354
Control	19 (82.6)	4 (17.4)	

[a] Numbers within parentheses represent percentages.
** $P<.01$, 1 d.f.

Since only one mouse was treated with 75 µg $PGF_2\alpha$, an accurate assessment of its effects cannot be made. A higher percentage of fetal resorptions occurred at the 37.5 µg treatment level than observed in Exp. A or B, although this increase did not differ from controls in this experiment. Average viable fetal weights were lower ($P<.05$), principally because viable fetuses were absent for the 300 and 75 µg treatment groups. No differences in average resorbed fetal weights or average total ovarian weights occurred between treatments (table 11).

Experiment D. Pregnant mice receiving the same regimen as Exp. C were analyzed on either d 11, 12, 13 or 14 of gestation in order to determine the rate of embryonic mortality following $PGF_2\alpha$ administration. Because of insufficient numbers of mice used in this experiment, an accurate description of the rate of fetal resorption following treatment could not be made. However, as indicated in table 12, fetal resorption was evident 2 d following administration of 300 µg $PGF_2\alpha$ and 1 d following administration of 150, 75 and 37.5 µg $PGF_2\alpha$. Pooled fetal viability scores within treatments are presented in table 13. All $PGF_2\alpha$ treatments resulted in a higher ($P<.01$) percentage of fetal resorptions compared to controls. There were no differences in average viable fetal weights, average resorbed fetal weights or average total ovarian weights between treatments (table 14).

Experiment E, Parts 1 and 2. Since the active agent(s) within ponderosa pine needles have their mode of action via an oral route of administration, the effectiveness of orally administered prostaglandins

TABLE 11. LEAST-SQUARES MEANS FOR VIABLE FETAL WEIGHTS, RESORBED FETAL WEIGHTS AND OVARIAN WEIGHTS AS AFFECTED BY $PGF_2\alpha$ TREATMENT (EXPERIMENT C)

Item	$PGF_2\alpha$, µg/.1 ml										SD[a]
	300		150		75		37.5		0		
No. of mice	3		4		1		2		3		
Avg viable fetal wt, g	.0305[c]	(0)[d]	.2010[bc]	(13)	.0262[c]	(0)	.4440[b]	(13)	.5498[b]	(19)	.1727
Avg resorbed fetal wt, g	.0353	(23)	.0345	(24)	.0366	(8)	.0356	(7)	.0037	(4)	.0201
Avg ovarian wt, g	.0076	(3)	.0088	(4)	.0109	(1)	.0080	(2)	.0073	(3)	.0016

[a] Standard deviation derived from pooled error term.
[b,c] Means within rows followed by unlike superscripts differ ($P<.05$).
[d] Values within parentheses indicate number of observations per mean.

TABLE 12. FETAL VIABILITY AT DIFFERENT DAYS FOLLOWING $PGF_2\alpha$ TREATMENT (EXPERIMENT D)

Treatment	Day of gestation analyzed	No. of viable fetuses	No. of dead fetuses	Fetal viability, %
$PGF_2\alpha$, µg/.1 ml				
0 (control)	11	12	0	100
	12	10	1	91
	13	12	0	100
37.5	11	3	9	25
	12	7	6	54
	13	5	8	38
	14	22	4	85
75	11	0	13	0
	12	4	12	67
	13	12	0	100
150	11	0	10	0
	12	6	3	67
	14	0	9	0
300	12	0	10	0
	13	17	8	68
	14	1	9	10

TABLE 13. CHI-SQUARE ANALYSIS OF $PGF_2\alpha$ TREATMENT ON FETAL VIABILITY COMPARED TO CONTROLS (EXPERIMENT D)

Treatment	No. viable fetuses	No. dead fetuses	χ^2
$PGF_2\alpha$, µg/.1 ml			
300	18 (40)[a]	27 (60)	28.2328**
150	6 (21.4)	22 (78.6)	38.4863**
75	20 (54)	17 (46.0)	17.8100**
37.5	37 (57.8)	27 (42.2)	17.2587**
Control	34 (97.1)	1 (2.9)	

[a] Numbers within parentheses represent percentages.
** P<.01, 1 d.f.

TABLE 14. LEAST-SQUARES MEANS FOR VIABLE FETAL WEIGHTS, RESORBED FETAL WEIGHTS AND OVARIAN WEIGHTS AS AFFECTED BY $PGF_2\alpha$ TREATMENT (EXPERIMENT D)

Item	$PGF_2\alpha$, µg/.1 ml					SD[a]
	300	150	75	37.5	0	
No. of mice	4	3	3	5	3	
Avg viable fetal wt, g	.2273 (18)[b]	.0654 (6)	.1240 (20)	.2389 (37)	.1261 (34)	.1298
Avg resorbed fetal wt, g	.0584 (27)	.0439 (22)	.0465 (17)	.0416 (27)	.0034 (1)	.0312
Avg ovarian wt, g	.0138 (4)	.0122 (3)	.0101 (3)	.0119 (5)	.0105 (3)	.0033

[a] Standard deviation derived from pooled error term.
[b] Values within parentheses indicate number of observations per mean.

in terminating pregnancy must be determined before they can be implicated as causative agents in pine needle abortion. Only limited research on orally administered prostaglandins using laboratory animals has been conducted. This route of administration would subject prostaglandins to lipolytic enzymes, a higher percentage of breakdown products and a longer reaction time when compared to other routes of administration. This is evidenced by the requirement of higher concentrations of $PGF_2\alpha$ in producing comparable luteolytic response in laboratory animals (Goyings, 1979). Table 15 shows the effects of fetal viability following oral administration of PGE_2 and $PGF_2\alpha$. Oral administration of 1500 µg $PGF_2\alpha$ resulted in a significant increase ($P<.05$) in fetal resorptions compared to mice receiving phosphate buffer. A higher percentage of fetal resorptions was observed in mice treated with 750 µg $PGF_2\alpha$ and 1000 and

TABLE 15. CHI-SQUARE ANALYSIS OF $PGF_2\alpha$ AND PGE_2 TREATMENT ON FETAL VIABILITY COMPARED TO CONTROLS (EXPERIMENT E, PARTS 1 AND 2)

Treatment	No. viable fetuses	No. dead fetuses	χ^2
$PGF_2\alpha$, µg/.3 ml			
1500	157 (77.3)[a]	46 (22.7)	6.4925*
$PGF_2\alpha$, µg/.2 ml			
750	161 (85.2)	28 (14.8)	1.6513
PGE_2, µg/.2 ml			
1000	59 (90.8)	6 (9.2)	.0119
500	59 (89.4)	7 (10.6)	.1395
Control	63 (91.3)	6 (8.7)	

[a] Numbers within parentheses represent percentages.
* $P<.05$, 1 d.f.

500 µg PGE_2, although this increase was not different from controls. Average viable fetal weights, average resorbed fetal weights and average total ovarian weights were not different between treatments (table 16).

Experiment E, Part 3. The oral administration of abietic acid was predicted to serve as a comparison between toxic agents in terminating pregnancy vs the effect of a luteolytic agent such as prostaglandin. Chi-square analysis of fetal viability following abietic acid treatment is presented in table 17. Oral administration of 50 and 22.4 mg of abietic acid on d 12 of gestation caused a significantly higher ($P<.01$) percentage of fetal resorptions than observed in control mice receiving a similar volume of ethanol. Mice receiving 10 mg abietic acid had a numerically higher percentage of resorbed fetuses than controls, but this difference was not significant.

A very noticeable difference existed between viable and resorbed fetuses from mice treated with abietic acid compared to fetuses of prostaglandin-treated mice. Fetuses in the process of resorption following abietic acid treatment were white in color, with physical form still discernable and a considerable amount of fluid contained within fetal extraembryonic membranes. Other resorbed fetuses within the same uterus were characteristic of prostaglandin-treated mice. Fetuses were brown in color and had a nondistinguishable physical form. Average viable fetal weight of mice treated with 50 mg of abietic acid were significantly lower ($P<.05$) than that of mice receiving the 10-mg treatment and numerically lower than the remaining treatment groups. There was an observable difference in physical size of viable fetuses between

TABLE 16. LEAST-SQUARES MEANS FOR VIABLE FETAL WEIGHTS, RESORBED FETAL WEIGHTS AND OVARIAN WEIGHTS AS AFFECTED BY $PGF_2\alpha$ AND PGE_2 TREATMENT (EXPERIMENT E, PARTS 1 AND 2)

Item	$PGF_2\alpha$		PGE_2			
	µg/.3 ml	µg/.2 ml	µg/.2 ml			
	5000	750	1000	500	0	SD[a]
No. of mice	17	17	5	5	5	
Avg viable fetal wt, g	.7708 (157)[b]	.9633 (161)	1.0579 (59)	1.1112 (59)	.9553 (63)	.3513
Avg resorbed fetal wt, g	.0120 (46)	.0124 (28)	.0113 (6)	.0079 (7)	.0014 (6)	.0193
Avg ovarian wt, g	.0150 (17)	.0178 (17)	.0168 (5)	.0143 (5)	.0128 (5)	.0078

[a] Standard deviation derived from pooled error term.
[b] Values within parentheses indicate number of observations per mean.

TABLE 17. CHI-SQUARE ANALYSIS OF ABIETIC ACID TREATMENT ON FETAL VIABILITY COMPARED TO CONTROLS (EXPERIMENT E, PART 3)

Treatment	No. viable fetuses	No. dead fetuses	χ^2
Abietic acid, 10^4 µg/.2 ml			
5	54 (71.1)[a]	22 (28.9)	7.9288**
2.24	66 (64.7)	36 (35.3)	13.1163**
1	83 (81.4)	19 (18.6)	3.5271
Control	34 (94.4)	2 (5.6)	

[a] Numbers within parentheses represent percentages.
** P<.01, 1 d.f.

mice treated with 50 and 22.4 mg abietic acid compared to the remaining treatment groups (figure 3). These results were similar to those obtained by Kubik and Jackson (1981) who reported that embryos of mice treated with diterpene resin acids derived from ponderosa pine needles were smaller and weighed less than controls. Although two stages of resorption were apparent following abietic acid treatment, this was not reflected in average resorbed fetal weights (table 18). Average total ovarian weights were not different between treatments, although in some mice in which fetal resorption occurred ovaries were surrounded by a substantial amount of fluid.

Mice Experiments Testing Pine Needle Fractions for Prostaglandin Activity

The objectives of these experiments were to test the biological activity of three separate pine needle fractions from a prostaglandin purification scheme (figure 1) utilizing pregnant mice.

Figure 3. Effect of abietic acid treatment on size of fetus at d 17 of gestation.

Experiment 1, Fraction 1. Chi-square analysis of fetal viability following pine needle extract treatment is presented in table 19. Mice receiving the 80-mg pine needle extract treatment for a 5-d period had a higher ($P<.01$) percentage of fetal resorptions compared to controls administered phosphate buffer. Mice receiving the 40-mg pine needle extract treatment had a numerically higher percentage of fetal resorptions than controls, although this difference was not significant. The administration of the 20-mg pine needle extract treatment also resulted in increased ($P<.05$) fetal resorptions compared to controls. Average viable fetal weights were not different between treatment groups (table 20). Average total ovarian weights were higher ($P<.05$) in control mice compared to the 40-mg treatment group. This latter observation was

TABLE 18. LEAST-SQUARES MEANS FOR VIABLE FETAL WEIGHTS, RESORBED FETAL WEIGHTS AND OVARIAN WEIGHTS AS AFFECTED BY ABIETIC ACID TREATMENT (EXPERIMENT E, PART 3)

Item	Abietic acid, 10^4 µg/.2 ml								SD[a]
	5		2.24		1		0		
No. of mice	6		8		8		3		
Avg viable fetal wt, g	.5819[c]	(54)[d]	.6516[bc]	(66)	1.0062[b]	(83)	.7606[bc]	(34)	.2922
Avg resorbed fetal wt, g	.0519	(22)	.0236	(36)	.0150	(19)	.0025	(2)	.0423
Avg ovarian wt, g	.0140	(6)	.0171	(8)	.0198	(8)	.0140	(3)	.0045

[a] Standard deviation derived from pooled error term.
[b,c] Means within rows followed by unlike superscripts differ (P<.05).
[d] Values within parentheses indicate number of observations per mean.

TABLE 19. CHI-SQUARE ANALYSIS OF FRACTION 1 TREATMENT ON FETAL VIABILITY COMPARED TO CONTROLS (EXPERIMENT 1)

Treatment	No. viable fetuses	No. dead fetuses	χ^2
Fraction 1, 10^4 µg/.2 ml			
8	108 (75.2)[a]	29 (24.8)	7.2839**
4	124 (87.9)	17 (12.1)	.9349
2	105 (82.0)	23 (18.0)	4.5051*
Control	90 (91.8)	8 (8.2)	

[a] Numbers within parentheses represent percentages.
* P<.05, 1 d.f.
** P<.01.

not readily explained since the 40-mg treatment group had the second highest percentage fetal viability. Only two resorbed fetuses recovered from mice treated with 80 and 20 mg of pine needle extract were characteristic of those observed in abietic acid treatments. The remaining resorbed fetuses were nondistinguisable in form and brown in color. Two mice receiving the 80-mg treatment and one mouse receiving the 40-mg treatment contained mummified uteri upon examination on d 17 of gestation. Uteri were swollen and rubbery in texture and in each case all fetuses were resorbed with placentas adhered to uterine walls.

Experiment 2, Fraction 1. In Exp. 2, higher concentrations of pine needle extract were prepared and administered orally to pregnant mice for a 5-d treatment period beginning on d 12 of gestation. Only the 80-mg treatment resulted in a higher (P<.05) percentage of resorbed fetuses when compared to controls (table 21). Mice receiving the 120-mg treatment had a numerically higher percentage of resorbed fetuses compared

TABLE 20. LEAST-SQUARES MEANS FOR VIABLE FETAL WEIGHTS AND OVARIAN WEIGHTS AS AFFECTED BY FRACTION 1 TREATMENT (EXPERIMENT 1)

Item	Fraction 1, 10^4 µg/.2 ml								SD[a]
	8		4		2		0		
No. of mice	14		14		13		9		
Avg viable fetal wt, g	1.0890	(108)[d]	1.0383	(124)	1.0288	(105)	1.1223	(90)	.2491
Avg ovarian wt, g	.0148[bc]	(14)	.0127[c]	(14)	.0145[bc]	(13)	.0162[b]	(9)	.0032

[a] Standard deviation derived from pooled error term.
[b,c] Means within rows followed by unlike superscripts differ (P<.05).
[d] Values within parentheses indicate number of observations per mean.

TABLE 21. CHI-SQUARE ANALYSIS OF FRACTION 1 TREATMENT ON FETAL VIABILITY COMPARED TO CONTROLS (EXPERIMENT 2)

Treatment	No. viable fetuses	No. dead fetuses	χ^2
Fraction 1, 10^4 µg/.2 ml			
16	38 (90.5)[a]	4 (9.5)	.5300
12	38 (73.1)	14 (26.9)	1.3000
8	28 (62.2)	17 (37.8)	3.9669*
Control	22 (84.6)	4 (15.4)	

[a] Numbers within parentheses represent percentages.
* P<.05, 1 d.f.

to controls, but this difference was not significant. Surprisingly, mice administered the highest concentration of pine needle extract had the lowest percentage of fetal resorptions. Two mice receiving the 160-mg treatment contained fetuses which appeared normal except for a pale white color suggestive of an earlier stage of resorption than observed in abietic acid treatments. Furthermore, two mice receiving this treatment were lethargic and cold to the touch during the last 2 d of the treatment period. Neither average viable fetal weights nor average total ovarian weights were different between treatment groups (table 22). Results from Exp. 1 and 2 provide evidence for biological activity in the phosphate buffer extract of these ponderosa pine needles, with the greatest biological activity existing between the range of 20 to 80 mg/.2 ml delivery. The apparent decrease in biological activity in the 120- and 160-mg concentrations is not understood but may be due to exceeding the saturation range for the volume of solvent used in this experiment. Preparations at these two concentrations had to be

TABLE 22. LEAST-SQUARES MEANS FOR VIABLE FETAL WEIGHTS AND OVARIAN WEIGHTS AS AFFECTED BY FRACTION 1 TREATMENT (EXPERIMENT 2)

Item	Fraction 1, 10^4 µg/.2 ml				SD[a]
	16	12	8	0	
No. of mice	4	5	4	3	
Avg viable fetal wt, g	.8396 (38)[b]	.8816 (38)	1.0433 (28)	.3331 (22)	.3411
Avg ovarian wt, g	.0142 (4)	.0153 (5)	.0133 (4)	.0126 (3)	.0037

[a] Standard deviation derived from pooled error term.
[b] Values within parentheses indicate number of observations per mean.

thoroughly mixed prior to administration. Activity at this stage did not necessarily demonstrate prostaglandin activity, since other pine needle constituents such as diterpene resin acids were present in this fraction. In fact, resorbed fetuses found in this experiment were strikingly similar in appearance to those observed following treatments with abietic acid.

Experiment 1, Fraction 2. This experiment along with Exp. 2 and 3 tested the biological activity of fraction 2. This fraction as illustrated in figure 1 represented a purified pine needle extract prior to column separation. Chi-square analysis of fetal viability following administration of fraction 2 is presented in table 23. Mice receiving a single ip injection of 3378 µg of fraction 2 had a higher ($P<.01$) percentage of fetal resorptions compared to controls receiving a similar volume of DMSO. The 1689-µg treatment group had a higher ($P<.01$) percentage of viable fetuses compared to controls. All resorbed

TABLE 23. CHI-SQUARE ANALYSIS OF FRACTION 2 TREATMENT ON FETAL VIABILITY COMPARED TO CONTROLS (EXPERIMENT 1)

Treatment	No. viable fetuses	No. dead fetuses	χ^2
Fraction 2, µg/.1 ml			
3378	85 (68.5)[a]	39 (31.5)	10.3480**
1689	87 (98.9)	1 (1.1)	7.4220**
844.5	43 (91.5)	4 (8.5)	.2124
422.25	67 (93.1)	5 (6.9)	.7610
Control	64 (88.9)	8 (11.1)	

[a] Numbers within parentheses represent percentages.
** $P<.01$, 1 d.f.

fetuses examined in this experiment were brown in color and nondistinguishable in form. No difference in average resorbed fetal weight or average total ovarian weight between treatments was noted (table 24). One mouse receiving the 3378-µg treatment had a mummified uterus as observed in Exp. 1, fraction 1. Mice within the 3378-µg treatment group had significantly lower ($P<.05$) average viable fetal weights than mice receiving 844.5 µg of fraction 2 and controls receiving DMSO.

Experiments 2 and 3, Fraction 2. Fraction 2 was separated into buffer-soluble and DMSO-soluble subfractions to individually test their biological activity. Chi-square analysis of fetal viability following oral administration of DMSO-soluble fraction 2 is presented in table 25. There were no differences in fetal viability when compared to controls. Average viable fetal weights of mice receiving the 1076-µg treatment were numerically lower than the remaining treatment groups and significantly lower ($P<.05$) than mice receiving 2070 µg of fraction 2 (table 26). Average resorbed fetal weights and average total ovarian weights were not different between treatment groups. As indicated in table 27, fetal viability of mice receiving ip injections of buffer-soluble fraction 2 were not different from controls receiving a similar volume of phosphate buffer. Control mice had significantly lower ($P<.05$) average viable fetal weights than the remaining treatment groups. Furthermore, controls and mice receiving two treatments had lower ($P<.05$) average total ovarian weights than mice receiving three treatments of 1715 µg fraction 2 (table 28).

TABLE 24. LEAST-SQUARES MEANS FOR VIABLE FETAL WEIGHTS, RESORBED FETAL WEIGHTS AND OVARIAN WEIGHTS AS AFFECTED BY FRACTION 2 TREATMENT (EXPERIMENT 1)

Item	Fraction 2, µg/.1 ml										SD[a]
	3378		1689		844.5		422.25		0		
No. of mice	11		8		4		7		8		
Avg viable fetal wt, g	.6314[c]	(85)[d]	.9244[bc]	(87)	1.1397[b]	(43)	1.0312[bc]	(67)	1.1178[b]	(64)	.3563
Avg resorbed fetal wt, g	.0299	(39)	.0107	(1)	.0271	(4)	.0218	(5)	.0311	(8)	.0423
Avg ovarian wt, g	.0118	(11)	.0131	(8)	.0118	(4)	.0140	(7)	.0133	(8)	.0030

[a] Standard deviation derived from pooled error term.
[b,c] Means within rows followed by unlike superscripts differ (P<.05).
[d] Values within parentheses indicate number of observations per mean.

TABLE 25. CHI-SQUARE ANALYSIS OF FRACTION 2 TREATMENT ON FETAL VIABILITY COMPARED TO CONTROLS (EXPERIMENT 2)

Treatment	No. viable fetuses	No. dead fetuses	χ^2
Fraction 2, µg/.1 ml			
4140	80 (94.1)[a]	5 (5.9)	.9689
2070	32 (97.0)	1 (3.0)	1.5876
1076	47 (90.4)	5 (9.6)	.0162
Control	52 (89.7)	6 (10.3)	

[a] Numbers within parentheses represent percentages.
1 d.f.

TABLE 27. CHI-SQUARE ANALYSIS OF FRACTION 2 TREATMENT ON FETAL VIABILITY COMPARED TO CONTROLS (EXPERIMENT 3)

Treatment	No. of treat-ments	No. viable fetuses	No. dead fetuses	χ^2
Fraction 2, µg/.1 ml				
1715	3	12 (100)[a]	0 (0)	.3591
1715	2	58 (93.5)	4 (6.5)	.5857
1715	1	33 (86.8)	5 (13.2)	2.5714
Control	3	34 (97.1)	1 (2.9)	

[a] Numbers within parentheses represent percentages.
1 d.f.

TABLE 26. LEAST-SQUARES MEANS FOR VIABLE FETAL WEIGHTS, RESORBED FETAL WEIGHTS AND OVARIAN WEIGHTS AS AFFECTED BY FRACTION 2 TREATMENT (EXPERIMENT 2)

Item	Fraction 2, µg/.1 ml 4140	2070	1076	0	SD[a]
No. of mice	7	3	4	5	
Avg viable fetal wt, g	.3691[bc] (80)[d]	.4067[b] (32)	.3334[c] (47)	.3826[bc] (52)	.0356
Avg resorbed fetal wt, g	.0237 (5)	.0185 (1)	.0090 (5)	.0072 (6)	.0304
Avg ovarian wt, g	.0142 (7)	.0111 (3)	.0109 (4)	.0143 (5)	.0034

[a] Standard deviation derived from pooled error term.
[b,c] Means within rows followed by unlike superscripts differ (P<.05).
[d] Values within parentheses indicate number of observations per mean.

TABLE 28. LEAST-SQUARES MEANS FOR VIABLE FETAL WEIGHTS, RESORBED FETAL WEIGHTS AND OVARIAN WEIGHTS AS AFFECTED BY FRACTION 2 TREATMENT (EXPERIMENT 3)

Item	Fraction 2, no. of treatments at 1715 µg/.1 ml				SD[a]
	3	2	1	0	
No. of mice	1	5	3	3	
Avg viable fetal wt, g	.4404[b] (12)[d]	.3728[b] (58)	.3653[b] (33)	.1455[c] (34)	.0742
Avg resorbed fetal wt, g	.0026 (0)	.0120 (4)	.0071 (5)	.0064 (1)	.0136
Avg ovarian wt, g	.0173[b] (1)	.0124[c] (5)	.0141[bc] (3)	.0105[c] (3)	.0016

[a] Standard deviation derived from pooled error term.
[b,c] Means within rows followed by unlike superscripts differ (P<.05).
[d] Values within parentheses indicate number of observations per mean.

Experiment 1, Fraction 3. This experiment utilized pregnant mice in testing the biological activity of three fractions collected following column chromatographic separation (figure 1). Column fractions representing the prostaglandin A series, prostaglandin E series, prostaglandin F series and fraction 70/30 were administered orally to pregnant mice on d 12 and 13 of gestation. As shown in table 29, fetal viability of mice receiving prostaglandin-containing fractions was no different than controls. Following administration of fraction 70/30, three of the four mice subsequently aborted prior to examination at d 17 of gestation. The majority of aborted fetuses were consumed by female mice and a count of fetuses was not obtained. Therefore, fetal viability based on comparison to controls was not an accurate assessment of the biological activity of these fractions. Further chromatographic analysis of fraction 70/30 by Moses and Katherine Attrep, East Texas State University, indicated this fraction to contain a mixture of the

TABLE 29. CHI-SQUARE ANALYSIS OF FRACTION 3 TREATMENT ON FETAL VIABILITY COMPARED TO CONTROLS (EXPERIMENT 1)

Treatment	No. viable fetuses	No. dead fetuses	χ^2
Fraction 3, µg/.1 ml			
PGA (17070)	53 (82.8)[a]	11 (17.2)	.0238
PGE (74800)	48 (80.0)	12 (20.0)	.1451
PGF (58830)	53 (85.5)	9 (14.5)	.0060
Control (213790)	11 (84.6)	2 (15.4)	

[a] Numbers within parentheses represent percentages.
1 d.f.

prostaglandin A and E series. Average viable fetal weights and average total ovarian weights presented in table 30 were not different between treatments in this experiment. All resorbed fetuses observed within treatment groups were brown in color and had a nondistinguishable physical form.

In summarizing the results of tests using fractions 1, 2 and 3 of the prostaglandin purification scheme, there appeared to be a decrease in biological activity with increasing prostaglandin purification. The greatest abortifacient activity occurred in the crude extract preparation, although this aqueous fraction contained many pine needle constituents. Thus, activity at the 20- and 80-mg treatment levels was not positively indicative of prostaglandin activity. Fraction 2, "extract prior to column separation," contained acid-soluble lipids and additional pine needle constituents other than prostaglandins. Results following testing of fraction 2 indicated activity within DMSO-soluble fractions and slight activity in buffer-soluble fractions but only via ip injection. This would seem to indicate a loss of activity via the oral route, the requirement of higher concentrations than those used, and(or) confinement of activity in the buffer-soluble fraction. Dose-response data in testing fractions 1, 2 and 3 were not consistent with those obtained in $PGF_2\alpha$ dose-response experiments in that fetal resorptions did not necessarily increase with increasing concentrations. Since the control treatment in fraction 3 had such a high mortality rate, it was not possible to accurately assess the activity of the other test fractions. However, even when comparing fetal viability of these fractions to DMSO

TABLE 30. LEAST-SQUARES MEANS FOR VIABLE FETAL WEIGHTS AND OVARIAN WEIGHTS AS AFFECTED BY FRACTION 3 TREATMENT (EXPERIMENT 1)

Item	Fraction 3 (prostaglandin series), µg/.1 ml				SD[a]
	(A) 17070	(E) 74800	(F) 58830	(O) 213790	
No. of mice	6	5	6	3	
Avg viable fetal wt, g	1.0399 (53)[b]	1.1029 (48)	.9811 (53)	.4664 (11)	.3251
Avg ovarian wt, g	.0145 (6)	.0187 (5)	.0125 (6)	.0112 (3)	.0047

[a] Standard deviation derived from pooled error term.
[b] Values within parentheses indicate number of observations per mean.

controls in other experiments, only slight activity was indicated, especially in the PGF series.

Research by Goyings (1979) showing a decrease in pregnancy rate and mean number of fetuses per litter at oral administration of 600 µg $PGF_2\alpha$ per 30-g rat would indicate that a concentration of 58830 µg per dose of the PGF fraction should have been adequate to elicit a response in treated mice. Two things may have occurred. First, activity was lost during some stage of the purification process or, second, since the fractions were not totally purified, a higher concentration may have been required to elicit a response by oral administration. The decrease in average viable fetal weight observed in Exp. 1 and 2 of fraction 2 along with resorbed fetuses similar in appearance to those of abietic acid treatments, suggest the presence of diterpene resin acids in these ponderosa pine needle fractions.

With the exception of Exp. B, a decrease in average viable fetal weight between treatments in prostaglandin dose-response experiments was not observed. Furthermore, resorbed fetuses were uniform in appearance and weight throughout the treatment groups. The two observed stages of fetal resorption occurring in abietic acid treatment groups in contrast to the one stage of fetal resorption observed in prostaglandin dose-response experiments may indicate (1) two distinct modes of action in causing fetal resorptions and(or) (2) a slower mode of action in abietic acid-induced fetal resorptions.

Ovarian weight change did not consistently predict changes in fetal viability. Corpora lutea regression occurred in prostaglandin,

abietic acid and pine needle fraction treatments. However, corpora lutea loss was not reflected by changes in ovarian weight.

Results from these mice experiments do not provide conclusive evidence that prostaglandins are active agents in pine needle-induced abortions. However, the occurrence of abortions following administration of fraction 70/30 determined to contain the PGA and PGE series may merit further investigation. Since radioimmunoassay has been used to document the presence of prostaglandins in ponderosa pine needles, its use along with a bioassay such as the pregnant mouse would have aided in confirming the presence of prostaglandins throughout the purification process and in determining the concentrations of the purified products. An accurate assessment of the precise concentration of prostaglandins in ponderosa pine needles will be essential in determining the feasibility of prostaglandins as caustive agents in pine needle abortion.

Cattle Pine Needle Feeding Experiment

Following administration of pine needle diets (tables 5 and 6) seven of the eight experimental animals subsequently gave birth to viable and healthy calves at term. One calf was born during a snow storm and died 2 d later. Necropsy results indicated inadequate nursing as the probable cause of death. Assistance was required in delivering one calf due to a breech presentation, but the remaining calves were born normally and without assistance. Furthermore, there were no retained placentas following parturition.

The only observable effects from pine needle treatments occurred during the 5-d oral administration of pine needle pulp (table 5).

Cattle receiving this treatment developed diarrhea and were lethargic. This condition was neither observed in control animals receiving a similar volume of phosphate buffer nor did it occur during any other pine needle treatments. Most field observations report little indication of an impending abortion following pine needle consumption. However, Call and James (1978) reported cows became depressed and dull in appearance prior to aborting. Some ranchers have also reported a bloody discharge from the reproductive tract of cows eating pine needles. This was not observed in any of the cattle receiving pine needle treatments.

Stress conditions believed to predispose cattle to pine needle consumption and ultimately to the effects of pine needles in causing abortion were employed in indoor and outdoor pine needle feeding experiments. Both cows receiving pine needle diets at the indoor facilities and the control receiving a limited intake of prairie hay were declining in body condition throughout the 33-d experiment. Likewise, cattle fed pine needle diets at the outdoor facilities and corresponding controls receiving prairie hay were declining in body condition in addition to being subjected to the environmental stress imposed by winter weather.

Total and differential leukocyte counts during indoor and outdoor pine needle feeding experiments are presented in tables 31 and 32, respectively. All values were within a normal range determined by visual comparison with standard values reported by Schalm et al. (1975). The monitoring of leukocyte counts was anticipated to give some insight into a possible mode of action for causative agents in pine needle abortion. If infectious processes are involved in pine needle-induced

TABLE 31. TOTAL AND DIFFERENTIAL LEUKOCYTE COUNTS DURING INDOOR PINE NEEDLE FEEDING EXPERIMENT[a]

Cow no.	Treatment	No. of blood samples	WBC ($10^3/mm^3$) 4-12[b]	Differential count: Granulocytes (15-45)[c] 600-4000[d]	Band cells (0-2) 0-120	Lymphocytes (45-75) 2500-7500	Monocytes (2-7) 25-850	Eosinophils (2-20) 2-2400	Basophils (0-2) 0-200
				Prairie hay (d -2 to d 0)					
373	Prairie hay	1	7.4	1406	370	5180	296	74	74
379	Prairie hay	1	16.6	8632	332	7138	166	332	0
374	Prairie hay	1	18.0	9540	360	7560	180	360	0
377	Prairie hay	1	11.3	5198	565	4407	113	1017	0
				Chopped pine needles (d 1 to 4)					
373	Prairie hay	1	12.8	2048	512	9344	512	384	0
379	Prairie hay	1	16.2	4536	0	10530	0	1134	0
374	Pine needles	1	23.2	10208	232	9048	232	928	232
377	Pine needles	1	13.9	2224	556	10425	278	417	0
				Pine needle/prairie hay (d 5 to 7)					
373	Prairie hay	1	7.0	3010	280	3570	0	140	0
379	Prairie hay	1	6.7	1206	201	5092	0	201	0
374	Pine needles	1	6.1	1525	122	4148	0	305	0
377	Pine needles	1	7.8	3354	234	3354	0	858	0

TABLE 31 CONTINUED

Cow no.	Treatment	No. of blood samples	WBC ($10^3/mm^3$) 4-12[b]	Differential count: Granulocytes (15-45)[c] 600-4000[d]	Band cells (0-2) 0-120	Lymphocytes (45-75) 2500-7500	Monocytes (2-7) 25-850	Eosinophils (2-20) 2-2400	Basophils (0-2) 0-200
			Pine needle extract (d 11 to 15 and 17)						
373	Buffer	5	5.86 ± .22	2162 ± 210[e]	274 ± 69	3234 ± 245	155 ± 71	12 ± 12	0
379	Buffer	5	7.68 ± .32	2728 ± 155	263 ± 96	4257 ± 248	119 ± 63	232 ± 85	31 ± 19
374	Extract	5	5.35 ± .26	1660 ± 205	203 ± 20	3184 ± 328	114 ± 16	158 ± 57	0
377	Extract	5	7.36 ± .41	2712 ± 317	88 ± 74	3991 ± 294	217 ± 80	350 ± 125	0
			Pine needle pulp (d 28 to 33)						
373	Buffer	6	5.88 ± .41	2539 ± 409	81 ± 52	2931 ± 358	106 ± 40	227 ± 89	0
379	Buffer	6	8.68 ± .95	2585 ± 474	298 ± 152	5472 ± 878	22 ± 22	259 ± 55	0
374	Pulp	6	6.45 ± .75	2836 ± 380	202 ± 64	2743 ± 313	106 ± 61	564 ± 146	0
377	Pulp	6	7.22 ± .89	3074 ± 649	347 ± 118	2909 ± 448	53 ± 28	684 ± 233	0
			Prairie hay (d 34 to 38)						
373	Prairie hay	5	4.95 ± 1.15	2231 ± 570	110 ± 38	2480 ± 567	52 ± 25	75 ± 34	0
379	Prairie hay	5	6.98 ± .34	1849 ± 274	94 ± 32	4851 ± 436	27 ± 16	161 ± 53	0
374	Prairie hay	5	4.61 ± .26	1631 ± 315	82 ± 49	2608 ± 294	18 ± 11	269 ± 100	0
377	Prairie hay	5	5.55 ± .67	2111 ± 444	49 ± 13	2645 ± 482	60 ± 26	565 ± 91	0

[a] Cattle received pine needle preparations over a 32-d period corresponding to January 19 to February 19, 1983.
[b] Normal range of total white blood cell (WBC) count for bovine; absolute no. x $10^3/mm^3$.
[c] Normal range of each kind of WBC; relative %.
[d] Normal range of each kind of WBC/mm^3 based on total WBC count.
[e] Mean WBC count ± SE.

TABLE 32. TOTAL AND DIFFERENTIAL LEUKOCYTE COUNTS DURING OUTDOOR PINE NEEDLE FEEDING EXPERIMENT[a]

Cow no.	Treatment	No. of blood samples	WBC ($10^3/mm^3$) 4-12[b]	Differential count: Granulocytes (15-45)[c] 600-4000[d]	Band cells (0-2) 0-120	Lymphocytes (45-75) 2500-7500	Monocytes (2-7) 25-850	Eosinophils (2-20) 2-2400	Basophils (0-2) 0-200
372	Pine needles	5	6.25 ± .11[e]	2870 ± 412	128 ± 68	2232 ± 394	51 ± 37	827 ± 168	13 ± 13
378	Pine needles	5	5.12 ± .40	2259 ± 529	108 ± 49	2348 ± 475	30 ± 20	341 ± 70	30 ± 20
375	Prairie hay	5	4.72 ± .23	1233 ± 90	69 ± 21	3276 ± 234	50 ± 22	94 ± 20	0
376	Prairie hay	5	8.44 ± .49	3075 ± 266	180 ± 83	4172 ± 464	0	775 ± 267	48 ± 30

[a] Cattle were fed pine needle diets for a 26-d treatment period corresponding to February 8 to March 5, 1983.

[b] Normal range of total white blood cell (WBC) count for bovine; absolute no. x $10^3/mm^3$.

[c] Normal range of each kind of WBC; relative %.

[d] Normal range of each kind of WBC/mm^3 based on total WBC count.

[e] Mean WBC count ± SE.

abortions as suggested by Adams et al. (1979) and Neff et al. (1982), changes in leukocyte numbers should occur. In bacterial infections, the leukocyte number, especially neutrophils, may be increased greatly (leukocytosis). A decrease in leukocyte numbers (leukopenia) is encountered with bacterial endotoxins, septicemia and toxemia. Furthermore, decreases in both eosinophils and lymphocytes, used as a diagnostic measure of stress conditions, may be applicable in defining stress conditions involved in pine needle abortion. Although these hematological parameters are by no means specific for a particular infection and realizing that various factors such as the stress of handling animals and time of day when blood samples are taken also may influence leukocyte counts, it may still be of value in future experiments to note distinct changes in leukocyte counts during pine needle treatments as a potential diagnostic tool in defining pine needle abortion.

The objective of monitoring serum progesterone levels during pine needle treatment was to obtain preliminary data on the possibility of luteolytic agents, such as prostaglandins, as causative agents in pine needle abortion. Serum progesterone concentrations during indoor and outdoor pine needle feeding experiments are presented in tables 33 and 34, respectively. Because of the small number of experimental animals employed in this study and since there exists a rather wide range of progesterone levels within which pregnancy can occur normally, conclusions as to the variability between cows in this experiment cannot accurately be made. If luteolytic agents such as prostaglandins are

TABLE 33. SERUM PROGESTERONE CONCENTRATIONS DURING INDOOR PINE NEEDLE FEEDING EXPERIMENT

Treatment	Treatment period, d	Progesterone, ng/ml	
		Cow no. 377	Cow no. 374
Prairie hay	-2 to 0	4.79 (1)[a]	5.85 (1)
Chopped pine needles	1[b] to 4	7.32 (1)	4.35 (1)
Pine needles/prairie hay	5 to 7	6.97 (1)	3.72 (1)
Pine needle extract[c]	11 to 15 and 17	7.26 ± .93[d] (4)	4.10 ± .40 (4)
Pine needle pulp[c]	28 to 33	5.48 ± .69 (9)	2.81 ± .33 (11)
Prairie hay	34 to 38	4.37 ± .42 (5)	5.89 ± 1.44 (5)
		Cow no. 373	Cow no. 379
Prairie hay	-2 to 0	3.06 (1)	7.71 (1)
Prairie hay	1 to 4	2.55 (1)	5.28 (1)
Prairie hay	5 to 7	2.68 (1)	6.93 (1)
Prairie hay	11 to 15 and 17	4.20 ± .73 (4)	4.13 ± .71 (4)
Prairie hay	28 to 33	3.81 ± .31 (9)	4.22 ± .49 (10)
Prairie hay	34 to 38	3.63 ± .58 (5)	4.46 ± .67 (5)

[a] Values within parentheses represent number of blood samples per mean.
[b] Starts first day of pine needle treatment period, corresponding to January 19, 1983.
[c] Treatment administered orally via stomach tube.
[d] Mean ± SE.

TABLE 34. SERUM PROGESTERONE CONCENTRATIONS DURING OUTDOOR PINE NEEDLE FEEDING EXPERIMENT[a]

Treatment	Day of blood sampling	Progesterone/ng/ml	
		Cow no. 372	Cow no. 378
Pine needles/prairie hay	33[b]	7.57 (10)[c]	3.20 (10)
Pine needles/prairie hay	46	2.69 (23)	2.87 (23)
Pine needles/prairie hay	54	4.18 (35)	3.03 (35)
Pine needles/prairie hay	57	4.20 (35)	3.19 (35)
Pine needles/prairie hay	62	4.85 (0)	3.42 (0)
		Cow no. 375	Cow no. 376
Prairie hay	33	6.37	2.17
Prairie hay	46	6.06	4.33
Prairie hay	54	2.56	6.08
Prairie hay	57	4.03	5.36
Prairie hay	62	8.91	7.04

[a] All animals were fed prairie hay for a 32-d period prior to pine needle treatment period.
[b] Starts first day of pine needle treatment period, corresponding to February 8, 1983.
[c] Numbers within parentheses represent percentage of pine needles in diet at time of blood sampling.

involved in pine needle abortion, the concentrations contained within pine needle treatments in this experiment were not of an adequate level to cause corpus luteal regression and hence abortion. Following administration of a luteolytic dose of $PGF_2\alpha$, blood progesterone levels normally fall by one-half within 48 h and by 72 h may be below the limit of detection (Liehr et al., 1972; Inskeep, 1973; Chenault et al., 1976). This dramatic decrease in serum progesterone levels was not observed during these pine needle feeding experiments.

Call and James (1978) suggested that pine needle abortion may be more closely related to the stage of gestation during pine needle consumption rather than related to animal condition, the amount of pine needles consumed at a given time or the length of time that animals eat needles. Based on a 283-d gestation period and assuming that all calves were born at term, cattle in this experiment averaged 186 ± 6 d pregnant at the start of pine needle treatments. Research by Lauderdale (1974) indicated that heifers required a greater dose of $PGF_2\alpha$ and required a longer interval between injection to abortion when injected after 150 d as compared to injections made prior to 150 d of pregnancy. Furthermore, data derived from dose titration studies conducted by The Upjohn Company (McAllister, 1979) showed limited response in inducing abortions in heifers with 100 mg $PGF_2\alpha$ injected im during d 150 to 250 of pregnancy but a high abortion rate late in pregnancy when injected from d 250 to 277 of pregnancy.

As with the mice experiments, results from cattle experiments cannot conclusively implicate luteolytic compounds as causative agents in pine needle abortion. The fact that these cattle did not abort may indicate the stage of gestation at which pine needles are consumed could influence the ultimate effect of pine needle consumption on pregnancy. Since field observations and controlled experiments have not provided enough data to precisely determine the time in gestation when pine needle abortion occurs most often in cattle, it would be of interest to note whether a decreased susceptibility to pine needle abortion or longer interval from consumption to abortion occurs during the time

period when an ovarian source of progesterone is not as critical in maintaining pregnancy. This time period is believed to occur between d 150 to 250 of gestation. Data reported by Call and James (1976), which showed no effect on pregnancy in sheep when pine needles were fed during d 60 to 90 of gestation when an ovarian source of progesterone is not required to maintain pregnancy in this species, could suggest that a decreased susceptibility to pine needle abortion may exist during this "placenta shift" in cattle.

Assuming that luteolytic agents are involved in pine needle abortion, their effects could be potentiated by factors such as weather and nutritional stress via the release of adrenocorticotrophic hormone (ACTH). A large number of physical stresses can lead to greatly enhanced secretion of ACTH and consequently to corticosteroid production. During normal parturition elevated fetal corticosteroid levels stimulate the synthesis and release of $PGF_2\alpha$ by the maternal cotyledons and later by the myometrium (Hansel and McEntee, 1977). Increasing $PGF_2\alpha$ levels in turn cause corpus luteal regression leading to a fall in progesterone levels prior to parturition. In a field trial conducted by Barth et al. (1981), the efficacy of a combined $PGF_2\alpha$ analogue (cloprostenol) and a synthetic corticosteroid (dexamethasone) was tested as an abortifacient in feedlot heifers. Results indicated that this combined treatment would induce abortion at all stages of pregnancy in feedlot heifers at a higher incidence than cloprostenol or dexamethasone administered separately. Barth hypothesized that $PGF_2\alpha$ affects the luteal source of progesterone and dexamethasone affects the placental source of progesterone, both of

which may need to be eliminated to induce abortion consistently. The increased production of maternal corticosteroid in association with stress conditions could influence the magnitude of effects of luteolytic pine needle constituents such as prostaglandins. Furthermore, since preliminary studies have indicated the concentration of luteolytic agents in ponderosa pine needles to be low and since a higher concentration would be required via an oral route of administration to cause abortion, the implication of luteolytic agents in pine needle abortion at this stage of research would indicate the necessity of other potentiating factors in the termination of pregnancy via pine needle consumption.

SUMMARY

Six dose-response experiments were conducted utilizing pregnant mice to determine the effects of $PGF_2\alpha$, PGE_2 and abietic acid on pregnancy. Results from these experiments were used for comparison in determining the biological activity of three separate pine needle fractions which were intermediate in a procedure designed to separate and isolate prostaglandins from ponderosa pine needles. Of special interest in these mice experiments was the effect of treatment on fetal viability, viable fetal weight, resorbed fetal weight and ovarian weight. In addition to mice experiments, eight pregnant cows in at least the fifth month of gestation were used in a ponderosa pine needle feeding experiment to obtain preliminary information on the effects of pine needle consumption, coupled with nutritional and environmental stress conditions, on pregnancy, progesterone levels and leukocyte numbers.

Dose-response experiments in which $PGF_2\alpha$ was injected ip on d 10 or 12 of gestation resulted in decreased fetal viability with increasing concentration. Differences in average viable fetal weight, average resorbed fetal weight and total ovarian weight between treatments were not consistently observed. $PGF_2\alpha$ administered orally on d 12 and 13 of gestation resulted in decreased fetal viability but only at a concentration of 1500 μg. There was no significant effect following oral PGE_2 treatments.

The oral administration of abietic acid was expected to serve as a comparison between toxic agents in terminating pregnancy vs luteolytic agents such as prostaglandins. Mice receiving 22.4 and 50 mg of abietic

acid on d 12 of gestation had a higher percentage of fetal resorptions than observed in control mice. Abietic acid treatments had a noticeable effect on the size of viable fetuses which was not observed in prostaglandin treatments. Furthermore, two distinct types of fetal resorption were observed in abietic acid-treated mice compared to one type in prostaglandin-treated mice. Although two types of resorption were apparent, no differences in average resorbed fetal weight between treatments were found. The different effects on fetal size and appearance of resorbed fetuses between abietic acid treatments and prostaglandin treatments may indicate (1) two distinct modes of action in causing fetal resorptions and(or) (2) a slower mode of action in abietic acid-induced fetal resorptions.

Results from mice experiments testing fractions 1, 2 and 3 of the prostaglandin purification scheme showed a decrease in biological activity with increasing purification. The greatest abortifacient activity occurred in the crude extract preparation. This fraction contained many pine needle constituents including diterpene resin acids, the presence of which was suggested by resorbed fetuses similar in appearance to those observed in abietic acid treatments. Tests involving fraction 2, "extract prior to column separation," indicated slight biological activity within DMSO-soluble treatments and buffer-soluble treatments but only via ip injection. This may suggest (1) a loss of activity via oral administration, (2) the requirement of higher concentrations and(or) (3) confinement of activity in the buffer-soluble fraction. Fraction 3, containing individual prostaglandin series

collected following column separation, showed limited biological activity. However, it was not possible to accurately asses the activity of this fraction since the control treatment (fraction 70/30) resulted in the highest fetal mortality. Further chromatographic analyses of this column fraction indicated the presence of the prostaglandin A and E series.

No abortions occurred following administration of pine needle preparations to pregnant cattle. Furthermore, pine needle treatments had no noticeable effect on leukocyte numbers or progesterone levels.

Overall, the results from the mice and cattle experiments provide equivocal but not conclusive evidence that prostaglandins are active agents in pine needle-induced abortions. However, since radioimmunoassay and chromatographic analyses have determined that prostaglandins are present in ponderosa pine needles, further research should be conducted. It would be particularly worthwhile to determine (1) the precise concentrations of prostaglandins in a given weight of pine needles during different seasons, (2) dose-response relationships of pine needle-derived prostaglandins in promoting abortions in cattle, (3) whether a decreased susceptibility to pine needle abortion or longer interval from consumption to abortion occurs during the time period when an ovarian source of progesterone is not as critical in maintaining pregnancy in the cow and (4) the relationship between environmental and nutritional stresses and the concentration of orally administered prostaglandins required to cause abortion in pregnant cows.

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APPENDIX

TABLE 1. LEAST-SQUARES ANALYSIS OF VARIANCE FOR VIABLE FETAL WEIGHTS AND RESORBED FETAL WEIGHTS IN EXPERIMENT B

Source	df	Mean squares: Viable fetal wt	Mean squares: Resorbed fetal wt
Treatment	4	.6352895*	.0038340
Initial body wt	1	.0036440	.0159681**
Error	32	.2136780	.0016379
Total	37		

* P<.05.
** P<.01.

TABLE 2. LEAST-SQUARES ANALYSIS OF VARIANCE FOR OVARIAN WEIGHTS IN EXPERIMENT B

Source	df	Mean squares: Ovarian wt
Treatment	4	.0000062
Error	33	.0000182
Total	37	

TABLE 3. LEAST-SQUARES ANALYSIS OF VARIANCE FOR VIABLE FETAL WEIGHTS AND RESORBED FETAL WEIGHTS IN EXPERIMENT C

Source	df	Mean squares: Viable fetal wt	Mean squares: Resorbed fetal wt
Treatment	4	.1424042*	.0004864
Initial body wt	1	.0140329	.0001457
Error	7	.0298131	.0004044
Total	12		

* P<.05.

TABLE 4. LEAST-SQUARES ANALYSIS OF VARIANCE FOR OVARIAN WEIGHTS IN EXPERIMENT C

Source	df	Mean squares Ovarian wt
Treatment	4	.0000031
Error	8	.0000027
Total	12	

TABLE 5. LEAST-SQUARES ANALYSIS OF VARIANCE FOR VIABLE FETAL WEIGHTS AND RESORBED FETAL WEIGHTS IN EXPERIMENT D

		Mean squares	
Source	df	Viable fetal wt	Resorbed fetal wt
Treatment	4	.0203203	.0012780
Initial body wt	1	.0074004	.0000915
Error	12	.0168425	.0009718
Total	17		

TABLE 6. LEAST-SQUARES ANALYSIS OF VARIANCE FOR OVARIAN WEIGHTS IN EXPERIMENT D

Source	df	Mean squares Ovarian wt
Treatment	4	.0000075
Error	13	.0000110
Total	17	

TABLE 7. LEAST-SQUARES ANALYSIS OF VARIANCE FOR VIABLE FETAL WEIGHTS AND RESORBED FETAL WEIGHTS IN EXPERIMENT E, PARTS 1 AND 2

Source	df	Mean squares: Viable fetal wt	Mean squares: Resorbed fetal wt
Treatment	4	.1651304	.0001309
Initial body wt	1	.2939631	.0025561*
Error	43	.1234290	.0003735
Total	48		

* P<.05.

TABLE 8. LEAST-SQUARES ANALYSIS OF VARIANCE FOR OVARIAN WEIGHTS IN EXPERIMENT E, PARTS 1 AND 2

Source	df	Mean squares: Ovarian wt
Treatment	4	.0000346
Error	44	.0000606
Total	48	

TABLE 9. LEAST-SQUARES ANALYSIS OF VARIANCE FOR VIABLE FETAL WEIGHTS AND RESORBED FETAL WEIGHTS IN EXPERIMENT E, PART 3

Source	df	Mean squares: Viable fetal wt	Mean squares: Resorbed fetal wt
Treatment	3	.2552297	.0021870
Initial body wt	1	.1935899	.0001136
Error	20	.0853591	.0017915
Total	24		

TABLE 10. LEAST-SQUARES ANALYSIS OF VARIANCE FOR OVARIAN WEIGHTS IN EXPERIMENT E, PART 3

Source	df	Mean squares Ovarian wt
Treatment	3	.0000472
Error	21	.0000204
Total	24	

TABLE 11. LEAST-SQUARES ANALYSIS OF VARIANCE FOR VIABLE FETAL WEIGHTS IN EXPERIMENT 1, FRACTION 1

Source	df	Mean squares Viable fetal wt
Treatment	3	.0212893
Initial body wt	1	.1010174
Error	45	.0620426
Total	49	

TABLE 12. LEAST-SQUARES ANALYSIS OF VARIANCE FOR OVARIAN WEIGHTS IN EXPERIMENT 1, FRACTION 1

Source	df	Mean squares Ovarian wt
Treatment	3	.0000247
Error	46	.0000102
Total	49	

TABLE 13. LEAST-SQUARES ANALYSIS OF VARIANCE FOR VIABLE FETAL WEIGHTS IN EXPERIMENT 2, FRACTION 1

Source	df	Mean squares: Viable fetal wt
Treatment	3	.1625084
Initial body wt	1	.3687614
Error	11	.1163199
Total	15	

TABLE 14. LEAST-SQUARES ANALYSIS OF VARIANCE FOR OVARIAN WEIGHTS IN EXPERIMENT 2, FRACTION 1

Source	df	Mean squares: Ovarian wt
Treatment	3	.0000055
Error	12	.0000134
Total	15	

TABLE 15. LEAST-SQUARES ANALYSIS OF VARIANCE FOR VIABLE FETAL WEIGHTS AND RESORBED FETAL WEIGHTS IN EXPERIMENT 1, FRACTION 2

Source	df	Mean squares: Viable fetal wt	Resorbed fetal wt
Treatment	4	.3604202*	.0005691
Initial body wt	1	.0567930	.0000906
Error	32	.1269384	.0017924
Total	37		

* P<.05.

TABLE 16. LEAST-SQUARES ANALYSIS OF VARIANCE FOR OVARIAN WEIGHTS IN EXPERIMENT 1, FRACTION 2

Source	df	Mean squares Ovarian wt
Treatment	4	.0000070
Error	33	.0000089
Total	37	

TABLE 17. LEAST-SQUARES ANALYSIS OF VARIANCE FOR VIABLE FETAL WEIGHTS AND RESORBED FETAL WEIGHTS IN EXPERIMENT 2, FRACTION 2

		Mean squares	
Source	df	Viable fetal wt	Resorbed fetal wt
Treatment	3	.0029440	.0003347
Initial body wt	1	.0002577	.0036429
Error	14	.0012666	.0009272
Total	18		

TABLE 18. LEAST-SQUARES ANALYSIS OF VARIANCE FOR OVARIAN WEIGHTS IN EXPERIMENT 2, FRACTION 2

Source	df	Mean squares Ovarian wt
Treatment	3	.0000153
Error	15	.0000116
Total	18	

TABLE 19. LEAST-SQUARES ANALYSIS OF VARIANCE FOR VIABLE FETAL WEIGHTS AND RESORBED FETAL WEIGHTS IN EXPERIMENT 3, FRACTION 2

Source	df	Mean squares: Viable fetal wt	Mean squares: Resorbed fetal wt
Treatment	3	.0414426*	.0000347
Initial body wt	1	.0043311	.0003665
Error	7	.0055023	.0001862
Total	11		

* P<.05.

TABLE 20. LEAST-SQUARES ANALYSIS OF VARIANCE FOR OVARIAN WEIGHTS IN EXPERIMENT 3, FRACTION 2

Source	df	Mean squares: Ovarian wt
Treatment	3	.0000138*
Error	8	.0000025
Total	11	

* P<.05.

TABLE 21. LEAST-SQUARES ANALYSIS OF VARIANCE FOR VIABLE FETAL WEIGHTS IN EXPERIMENT 1, FRACTION 3

Source	df	Mean squares: Viable fetal wt
Treatment	3	.2143800
Initial body wt	1	.1855875
Error	15	.1056788
Total	19	

TABLE 22. LEAST-SQUARES ANALYSIS OF VARIANCE FOR OVARIAN WEIGHTS IN EXPERIMENT 2, FRACTION 3

Source	df	Mean squares Ovarian wt
Treatment	3	.0000486
Error	16	.0000222
Total	19	